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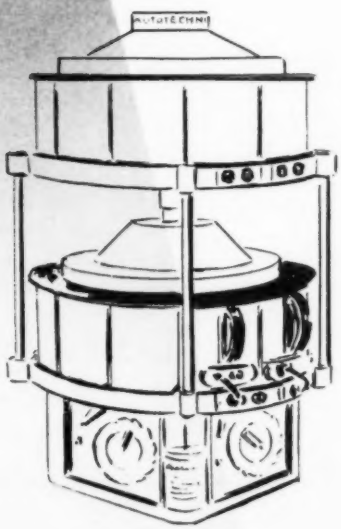

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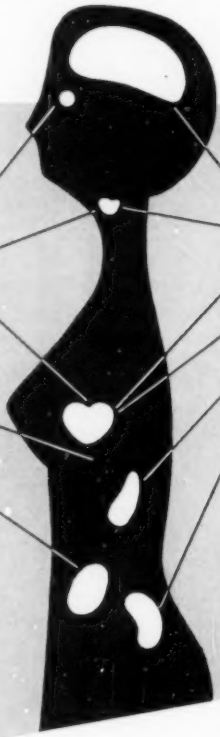

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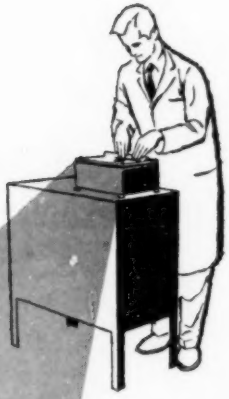

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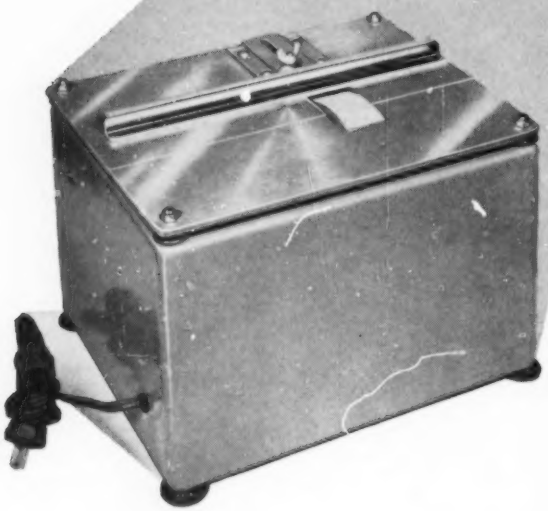

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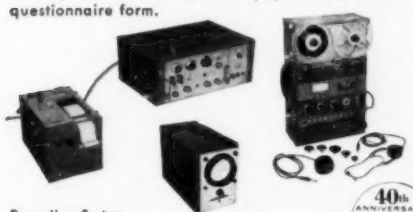

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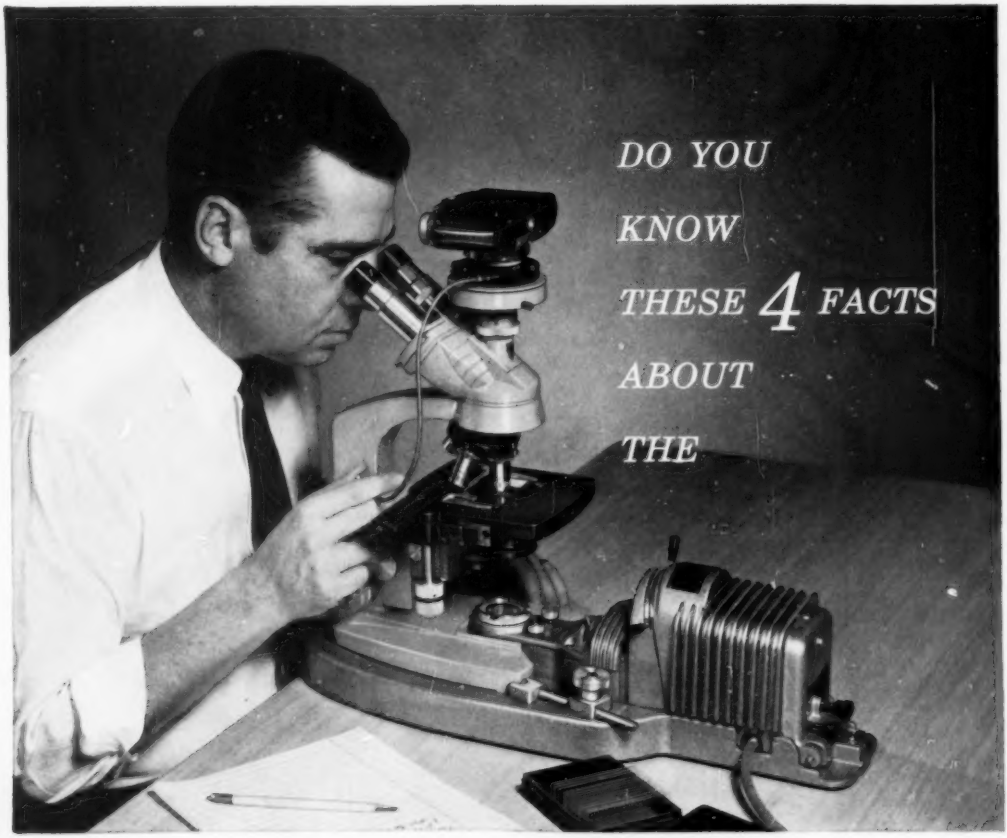

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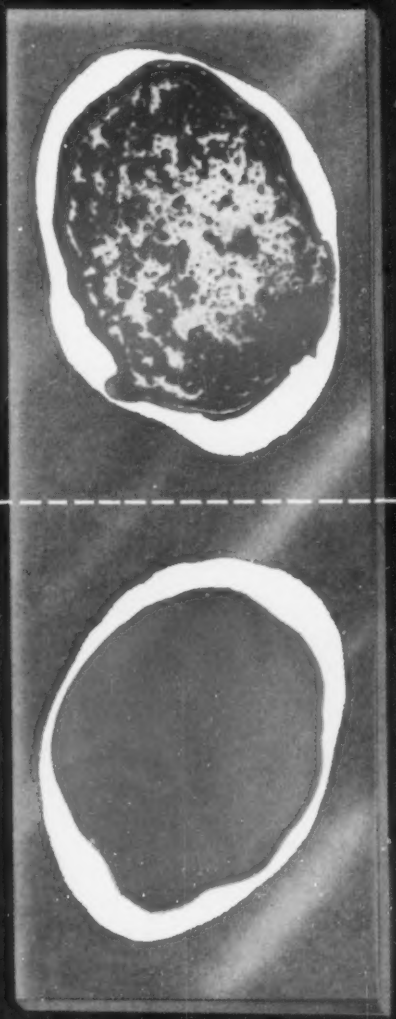

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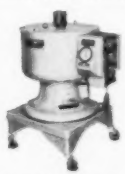

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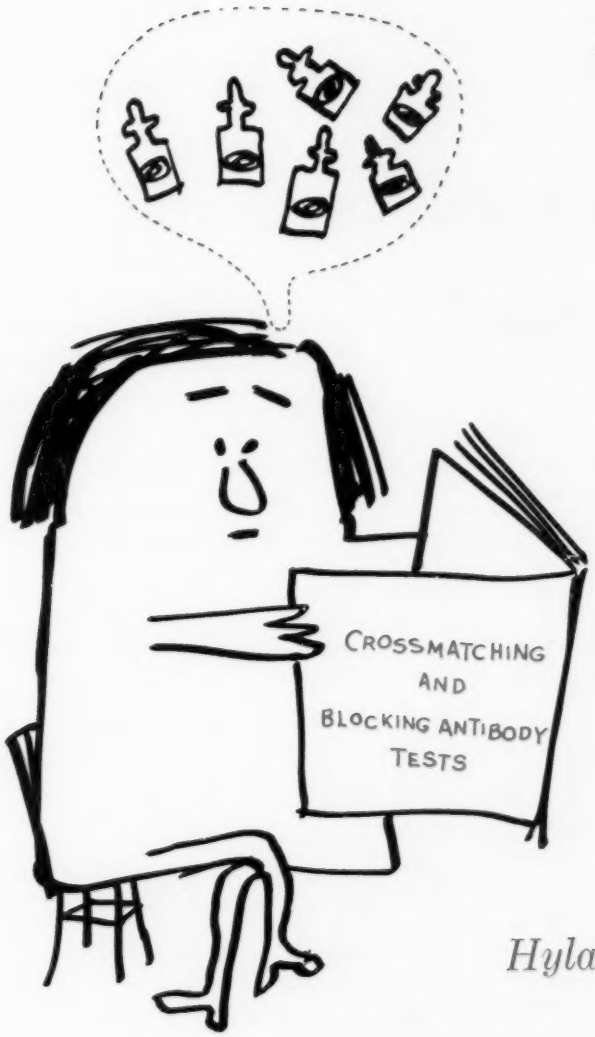

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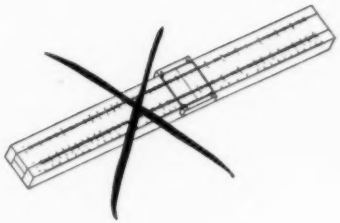

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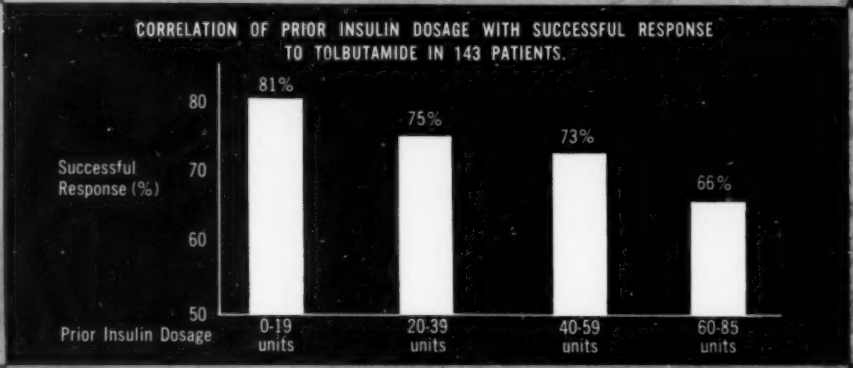

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Source—Sugar, S. J. N.: Ann. New York Acad. Sc. *71*:256 (July 10) 1957.

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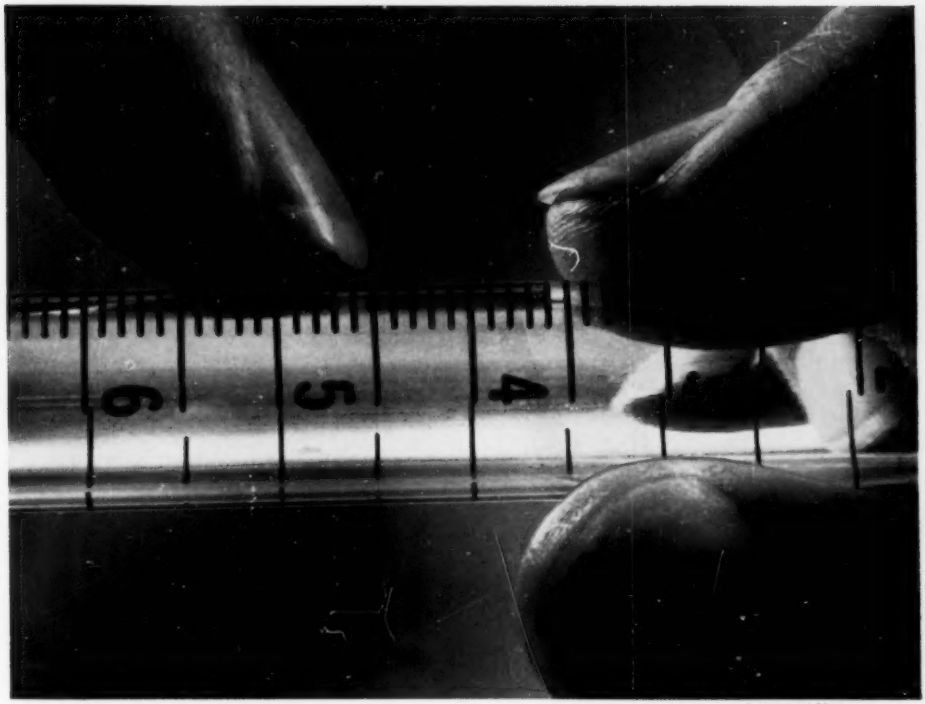

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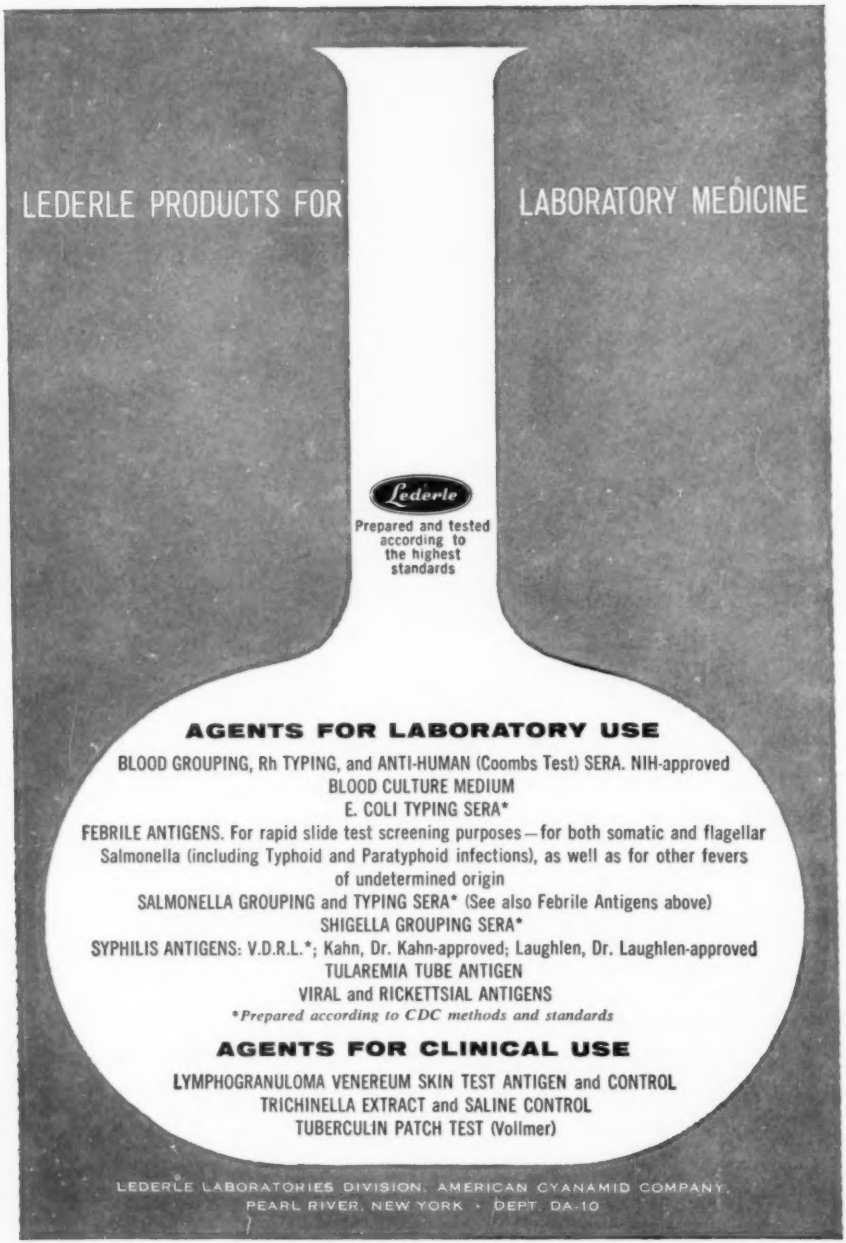
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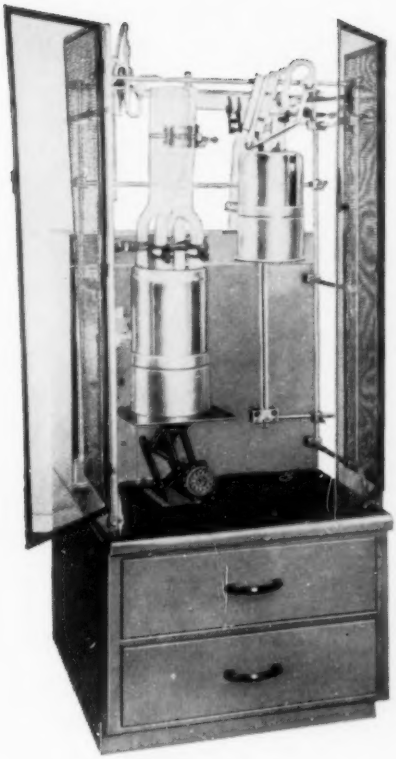

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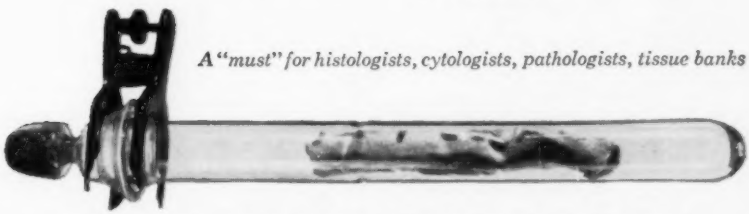

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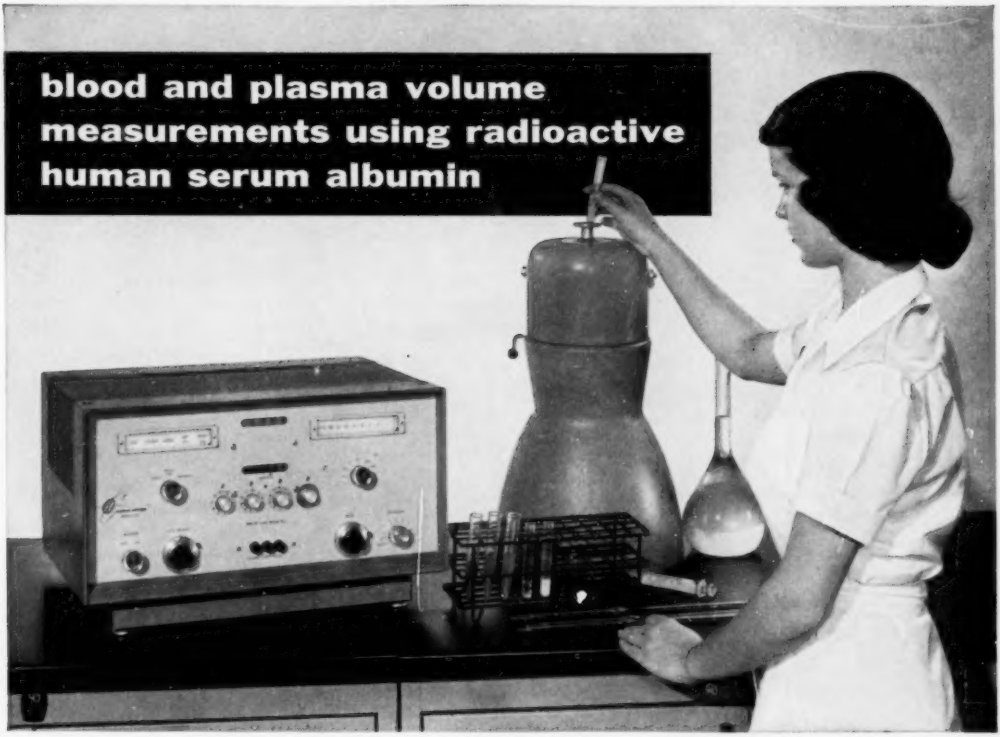

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The Interpretation of the
UNIPOLAR ELECTROCARDIOGRAM

by

GORDON B. MYERS, M.D.
Professor of Medicine
Wayne University College of Medicine
Head, Department of Medicine
City of Detroit Receiving Hospital

164 pages (8½" × 11")
Price, $4.75

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The Relation of the Electrocardiographic Deflections to Mechanical and Electrical Events in the Cardiac Cycle.

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* * *

C.
Origin and Form of the Normal and Abnormal QRS-T Complex.

* * *

D.
Disorders of Rate and Rhythm.

* * *

E.
Summary of QRS-T Patterns in Unipolar Thoracic and Limb Leads.

This work is the outgrowth of previous manuals prepared to supplement the Graduate Course in Electrocardiography given at Wayne University College of Medicine for many years. The objective is an organized presentation of the interpretation of the unipolar electrocardiogram. The only basic physics included is that considered essential to the interpretation of the tracing; controversial theories that do not contribute to clinical analysis have been omitted.

The analysis of the arrhythmias is made from leads that clearly display auricular as well as ventricular deflections; the interpretation of the QRS-T complex is based upon the findings in multiple precordial and back leads, supplemented, when necessary, by esophageal and unipolar limb leads. The registration of the normal and abnormal QRS-T complex is based upon concepts developed by Frank N. Wilson and associates and shown to be consistent with experimental and post-mortem findings. These concepts have been presented in detail and suitably illustrated with diagrams as an introduction to clinical analysis. Efforts have been concentrated on a systematic lucid description of electrocardiographic findings in an attempt to make reproduction of tracings unnecessary.

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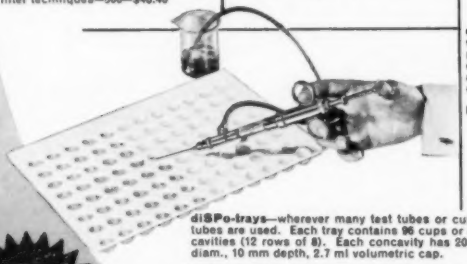

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This technique teeters on the brink of usefulness. Details are described in an article obtainable from Eastman Kodak Company, X-ray Division, Rochester 4, N. Y. It was written by Albert G. Richards, an associate professor of dentistry at the University of Michigan. If anybody else has ever used the method, we extend to him our heartiest congratulations. ☺

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radiography for topography... keeping faith with the medical technologists... what every practical-minded photomicrographer should know

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Up two bits

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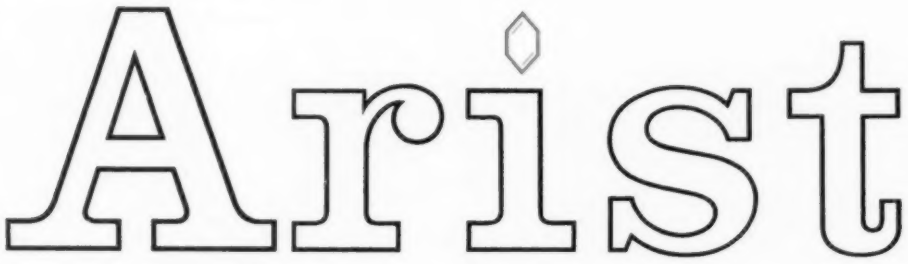

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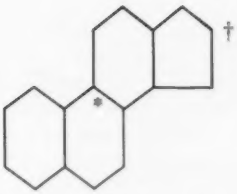

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*REFERENCES: Zoll, P. M., et al.: Termination of Ventricular Fibrillation in Man by Externally Applied Electric Countershock. New England Journal of Medicine, 254: 727, April 19, 1956. Zoll, P. M., et al.: The Effects of External Currents on the Heart. Circulation, 14: 745-756, November, 1956.

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LETTER FROM THE EDITORS

Dr. S. Howard Armstrong, Dr. A. C. Corcoran, and Dr. Randall G. Sprague have completed their six-year terms on the Board of Editors. On behalf of the Central Society for Clinical Research and the Editorial Board, we wish to express our appreciation for their valuable services.

Dr. William H. Daughaday, Dr. Robert A. Good, and Dr. Donald W. Seldin have accepted appointments on the Board of Editors.

During the past year the Editors and Board members have called upon many of the associates and colleagues in other universities to aid them in the evaluation of manuscripts. Those who have participated in this capacity in the past year are:

Albert, Alexander
Alpern, Mathew
Baker, Burton L.
Bauer, Jere M.
Berson, Solomon A.
Bishop, Ronald C.
Bohr, David F.
Bollett, Jay
Bolt, Robert J.
Braude, Abraham I.
Bricker, Neal
Carr, Edward A., Jr.
Chandler, Joseph P.
Christensen, Halvor N.
Christman, Adam A.
Cole, Jack
Conn, Jerome W.
Coon, Minor J.
Craig, Donald E.
Davenport, Fred M.
Davenport, Horace W.
Davey, Winthrop N.
Davidson, Charles S.
DeJong, Russell N.
DeWeese, Marion
Dodson, Vernon N.
Dunstan, Harriet
Dziewiatkowski, Dominic
Eckstein, Henry C.
Fajans, Stefan S.
Falls, Harold F.
Field, Henry, Jr.
Figley, Melvin
Fleisher, G. A.
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Gutman, Alexander B.
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Jacobson, Leon O.
Johnston, Franklin D.
Kark, Robert M.
Katz, Louis N.
Kerby, Grace
Korst, Don
Lapides, Jack
Lewis, Lena
Lewis, Jessica
Loosli, Clayton G.
McCullagh, Ernest P.
McIsaac, William
Manger, William
Mathews, Kenneth P.
Mattox, Vernon R.
Meyers, Muriel
Mickelson, Olaf
Moore, Carl V.
Narahara, Hiromicki
Necheles, Thomas
Neel, James
Pick, Ruth
Price, J. Waide
Pullman, Theodore
Ragan, Charles
Reiffel, Leonard
Riley, Gardner
Robertson, W. V.
Roseman, Saul
Ruffin, Julian
Salassa, R. M.
Schilling, Robert F.
Schroeder, Henry A.
Seldin, Donald W.
Simons, Charles
Sloan, Herbert E.
Soloman, A. K.
Stare, Fred
Stollerman, Gene H.
Storaasli, John
Streeten, David H. P.
Swell, Leon
Talbott, John
Taplin, George V.
Tsao, M. U.
Vilter, Carl F.
Watson, Cecil J.
Weller, John
Wheeler, A. H.
Wiggins, Hugh S.
Wilkinson, C. F.
Will, John
Williams, Robert H.
Winzler, R. J.
Wolff, Harold G.
Woods, Lauren A.
Wyngaarden, James B.
Ziff, Morris
Zinneman, Horace
Zuelzer, Wolf W.

We wish to take this opportunity to thank the past and present members of the Editorial Board for their excellent services to the JOURNAL and to the Central Society for Clinical Research. We would also like to express our appreciation for the cooperation received from The C. V. Mosby Company.

WILLIAM D. ROBINSON, M.D., EDITOR
WILLIAM H. BEIERWALTES, M.D., ASSOCIATE EDITOR

CLINICAL RESEARCH—A CAREER

KENNETH G. KOHLSTAEDT, M.D.*
INDIANAPOLIS, IND.

IT IS a great honor to preside at this, the thirtieth meeting of the Central Society. I wish to express my appreciation for the work of our excellent secretary, Dr. William Jordan, and for the cooperation and diligence of the Council. Each year the task of selecting thirty-six papers for presentation has become increasingly difficult. Limiting the number of abstracts that may be submitted by any one member has produced only a slight decrease in the total received.

The large number of well-qualified young men proposed for membership in 1957 indicates that within the geographical boundaries of the Central Society the future for clinical research and academic medicine is very bright.

Precedent decrees that at this point I have the privilege of addressing you on a subject of my own choice. With two exceptions, since 1949 the theme for the presidential address has dealt with some phase of medical research or education. I wish to continue the discussion of these all-important subjects.

We are all aware of the public interest in medical research. It has been whetted by articles in the magazines and by television shows which all too often portray only the glamorous and spectacular aspects. Even the *Wall Street Journal* reports new advances in therapy. Congress has responded by appropriating huge sums. "Crash programs" are popular because many persons believe that, given enough money, one can buy an answer to any problem. Voluntary health organizations have flourished and have come to play an important role in lay and professional education.

Acquiring new and bigger buildings with more gadgets and expensive equipment has become the goal of some investigators, and thereby they have allowed "the pure delight" of medical research, as it was so well described by Dr. Ed Allen,[1] to escape them forever. Professor Haldane,[2] of Oxford, used to say there were three stages in the career of any great man of science. In the first he did some notable piece of work, often in the corner of a cramped and sparsely fitted laboratory. In the second he spent his time planning a new and splendid building wherein he proposed to carry on and extend his researches. In the third he spent most of his time showing visitors around his fine laboratories. Adequate financial support and adequate laboratory space are essential, but these are of no value unless they are used by competent, well-trained investigators who are in turn supported by an adequate number of technical assistants, the "para-medical staff."

Presidential address, Thirtieth Annual Meeting of the Central Society for Clinical Research, Chicago, Illinois, Nov. 1 and 2, 1957.

*Director, Clinical Research Division, Eli Lilly and Company, and Professor of Medicine, Indiana University School of Medicine, Indianapolis, Ind.

In 1956, in his presidential address, Dr. Clayton Loosli[3] discussed medical education and training in research. He called attention to the need for formal training in the "art of scientific investigation." Dr. Loosli noted that this subject receives little attention from our professional medical educators. In my opinion this problem deserves our thought, and I would like to consider what we might do to stimulate interest in clinical research and teaching as a career. Research and teaching must always be considered as a single entity. Dr. Willard Rappleye,[4] Dean of Columbia University School of Medicine, has said, "Research is the life blood of medical education and progress. Efforts to separate them are both unrealistic and illogical."

The major effort in most educational programs for resident physicians is presently directed toward preparation for practice. Many persons assume that somehow (perhaps by "osmosis") training for clinical research will be acquired during a period of hospital service.

Today we frequently hear the complaint that resident physicians have only one interest—getting information that will enable them to pass an examination for certification by a board. The Board of Internal Medicine has done an excellent job in devising examinations that test both knowledge and skill. It is difficult, however, to evaluate the true competence of a physician by his ability to pass an oral and written examination. Dr. Alan Gregg,[5] a man who in his lifetime had broad experience in viewing medical education and research programs in many parts of the world, stated in his Bampton Lectures at Columbia in 1953, "The examiner does not find out what the candidate knows. He only finds whether the candidate knows what the examiner knows." The danger of an examination is that originality and freedom to go beyond the knowledge of the old "practitioner" may not be adequately encouraged. Dr. Gregg proposes that the task of rewarding originality and mature competence be entrusted to full-time teachers and investigators.

What can we offer as an alternative for board examinations? Perhaps a degree patterned after a Ph.D. in the preclinical science could be conferred on the physician who has proved to his faculty that he was a competent clinician and experienced scientific investigator. In some institutions a Doctor of Medical Science or Ph.D. degree in a special field of medicine already exists—or at least is described in the catalog. In some instances such a degree may have been awarded for an extension of resident training.

I would propose that a program be developed with great care, so that the quality of graduate training would be equal to that required for a Ph.D. degree in a major science such as chemistry, mathematics, or physics. Collaboration between the faculty of the medical school or research institution and persons in the administration of graduate education would be desirable.

In addition to experience with patients, the trainee for clinical research must have time for laboratory science. Experience has proved that these must occur at separate periods. The individual who attempts to work in a laboratory and at the same time serve as resident physician usually does neither assignment well. It would be difficult to cram all of the training needed for clinical

research into the period now required for a medical resident to qualify for board examination. If we include two years of military service, twelve or thirteen years are needed to complete the program; therefore, it would not be feasible to add training in research after completion of the residency.

I believe we should begin the program at the undergraduate level. Recently, leaders in medical education have given serious consideration to "telescoping" the premedical and medical programs. I would propose that we consider *integrating* the last two years of medical school with a program aimed at providing training in clinical research after graduation. Experience in research and contact with clinical investigation is now being made available in some medical schools. In one institution with which I am familiar, a quarter at the end of the junior year and the first quarter of the senior year can be set aside for students for a research project.

A criticism which is frequently voiced when an attempt is made to place medical students in contact with persons doing research is that "scientific training dries up the milk of human kindness." Dr. Dana Atchley[6] remarked that he had never seen a sympathetic person frozen by a research career, nor a cold, tactless individual thawed by general practice.

A unified program involving the junior and senior years of medical school and the internship and three years of residency could be so organized that time for a period in physiology or chemistry could be provided. In one midwestern university the resident in medicine may spend a whole year in the physiology department. During internship, time for instruction in other phases of training, i.e., medical statistics, could be included without seriously impairing the clinical work.

Excellent programs could be devised, but a greater flexibility in the medical curriculum than now generally exists would be needed. In some schools there are few electives. In a medical school with which I am associated, 150 students in each class receive the same training in the junior and senior years regardless of their plans for the future.

It would also be unwise to make experience in research compulsory. Dr. Dana Atchley,[6] in his address at the 53rd Annual Congress on Medical Education and Licensure, emphasized that "no manipulation of the curriculum and no brilliance of pedagogy can compare with participation in research for the production of a scientific critique." He also said, "Nothing is more futile than forced or contrived involvement in a research problem."

At the end of the second year, some students, with faculty advice and guidance, could be selected for a career in research, and the final decision would be based on the students' experience during the elective time allocated for a clinical research project during the junior and senior years.

Last year Dr. Randall Sprague[7] told this Society that the spirit of inquiry must be cultivated and that the fledgling investigator needs to be exposed not only to patient care and diagnosis, but to the scholarly discipline of the clinical investigator. Dr. Wesley Spink[8] has called it the "spirit of the inquiring

physician.'' He stressed the importance of knowledge of the natural history of disease as a prerequisite to clinical investigation, regardless of whether we are searching for a cause or evaluating a new therapeutic agent.

It is not possible to have one program that would be adaptable to all universities or research institutes. Certainly the interests of the student would need to be considered. Perhaps an advisor from the faculty or research staff would be given the task of developing the course for each student contemplating a career in medical research. Resident physicians participating in a plan such as I have outlined would have more than a superficial contact with research.

In some institutions participation by the resident in a research program may be *very* superficial. In fact he may be ''rewarded'' simply for a good job of case finding, drawing of blood samples, or writing orders for coded drugs by having his name placed on a paper as co-author. The study may have been well planned and executed, but this was accomplished by the senior investigators. If the ''research team'' produces a number of excellent and important contributions, the resident may acquire a substantial bibliography without ever having actually written a paper or developed an idea for research. The resident leaves the team to begin practice and, having decided he is a ''research expert,'' is eager to undertake any problem that is suggested. Because he lacks training in the art of investigation, his efforts may not produce satisfactory results. He is discouraged and unhappy. He may express bitterness toward those who had the good fortune to receive guidance and supervision and experience in all phases of research. Furthermore, a great deal of money can be wasted by the physician without training who is attempting to do research.

The physician may, in the course of a brief experience with a research group, learn a technique in which he becomes very proficient—he may be a ''pro'' rather than a professional man. This difference was described by Dr. Clifford Rassweiler,[9] President of the American Chemical Society. A ''pro'' is one who by mere practice or training and repeated performance has gained a high degree of skill in some specialized activity. This skill sets him apart from men who cannot match his accomplishment. The ''pro'' may lose his skill if he doesn't practice, but the work for which he is paid may supply all the practice required for maintenance of skill. He needs little or no studying. On the other hand, continuous ''self-education'' is the mark of distinction of the true ''professional man.'' His position and his compensation are based on the knowledge he has acquired. This must be kept up to date by reading and attending scientific meetings. He must use this knowledge to instruct others.

Finally, another reason for establishing a ''degree'' which could be awarded physicians who are trained investigators would be the medico-legal aspect of medical research. A man who has been guided and supervised by an experienced investigator will be cognizant of the moral responsibilities and the

rights of the patients or volunteers whom he may study in the course of an investigation. Should an unfortunate accident occur, the fact that the investigator had earned a degree for postgraduate training in clinical and laboratory medicine would be an asset in any legal difficulty that might arise.

Over a period of years I have visited many laboratories and have engaged in cooperative efforts with many clinical investigators. I am convinced there is no substitute, no short-cut for adequate, well-planned, and well-supervised training in clinical research.

This can be accomplished in a hospital or research institution not directly affiliated with a medical school. In the area of the Central Society there are several centers which have training programs that fulfill all aspects of the plan I have just reviewed. I hope my remarks may stimulate interest in the development of a program which will lead to the award of an advanced degree for proficiency in clinical research.

Today industry recognizes academic degrees in pay scales and in the selection of persons for advancement. If a "degree in clinical research" signified a *thorough* training in clinical medicine and the art of scientific investigation, it would be very helpful as a guide to those charged with allocation of funds for research or with selection of persons for appointment to the staff of a medical school or hospital interested in research and teaching. This recognition might stimulate young men and women to seek this degree in preference, perhaps, to certification as a specialist by a board examination.

The success of these programs depends on our wholehearted support. When we encourage young people to make clinical research a career, we are fulfilling the objectives of this Society, "to cultivate research and develop young men."

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CLINICAL AND EXPERIMENTAL

STUDIES ON THE NATURE OF THE PLASMA ERYTHROPOIETIC FACTOR(S)

JAMES W. LINMAN, M.D.,* FRANK H. BETHELL, M.D.,** AND MARTHA J. LONG, M.S.*
CHICAGO, ILL., AND ANN ARBOR, MICH.

THE existence of a humoral factor or factors with erythropoiesis stimulating activity has received considerable experimental confirmation in recent years. Although the chemical nature of the plasma erythropoietic factor is not known, some of its physical attributes have been described. We have found it to be stable over a wide range of temperature, acid soluble, destroyable by ashing, and not precipitable by perchloric acid. Ultraviolet absorption studies of "anemic" plasma have shown that although boiling and precipitation with perchloric acid removed 92.3 and 94.1 per cent of the materials absorbed at wave lengths of 260 and 280 mμ, respectively, the erythropoietic activity was not altered. The active factor is not removed by passage through either positive or negative ion exchange resin columns.

Preliminary experiments in our laboratory indicated that the factor was extractable with ether from boiled plasma extracts.[1] This report constitutes an extension of these studies concerning the ether solubility of the erythropoietic factor present in the plasma of phenylhydrazine induced anemic rabbits.

MATERIALS AND METHODS

Adult New Zealand rabbits were rendered anemic by the daily administration of 1 ml. of a 2.5 per cent solution of phenylhydrazine subcutaneously. After 6 to 7 such injections, with hemoglobin levels in every instance below 7 Gm. per cent, the animals were exsanguinated via the abdominal aorta and the plasma was immediately frozen pending preparation of the various extracts described below.

The plasma was divided into two portions. One aliquot was extracted 5 times with a volume of reagent ether equal to the original volume of plasma. The ether phase was then placed under reduced pressure to remove the ether and reconstituted to the original volume of the plasma with distilled water. This fraction was labeled "whole plasma—ether soluble." The water phase was boiled and precipitated with perchloric acid according to the method we have used before[2] and labeled "PFPE (protein-free plasma extract)—post-plasma ether extraction." PFPE was then prepared from the remaining plasma. This material was also divided into two parts. One portion was labeled PFPE and the other was extracted 5 times with an equal volume of reagent ether. The water phase was labeled "PFPE-ether insoluble" and the ether phase was evaporated to complete dryness. Reagent ether was then added in an amount equivalent to twice the original volume of plasma,

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filtered, and the filtrate was subjected to reduced pressure and all ether removed. A small amount of greenish oily material remained following this procedure which was reconstituted with distilled water to the original volume and labeled "PFPE-ether soluble."

Forty-eight female Carworth-Wistar rats weighing approximately 160 grams were divided into 2 groups of 12 rats and 4 groups of 6 rats. The groups of 12 rats were injected subcutaneously with amounts equivalent to 2 per cent of their body weight daily of the PFPE-ether soluble and PFPE-ether insoluble fractions for 2 weeks (total of 10 injections, Saturdays and Sundays excepted). Three groups of 6 rats received similar amounts of the other extracts listed above. The last group of 6 rats was given Ringer's solution and served as the control.

Hematologic studies consisted of hemoglobins, hematocrits, and erythrocyte and reticulocyte counts. All determinations were obtained prior to the injections and weekly thereafter except for the reticulocytes which were counted twice weekly throughout the experimental period of 4 weeks. Hemoglobins were determined by the cyanmethemoglobin photocolorimetric method, hematocrits by the microhematocrit technique, erythrocytes by hemacytometer, and the reticulocytes per 1,000 red cells were counted using dried brilliant cresyl blue coverslip films counterstained with Wright's stain. At the conclusion of the 2-week injection period, 4 rats in each group of 12 and 2 in each of the other groups were sacrificed and femoral marrow examined by the method previously employed.[2] The remaining animals were followed for an additional 2 weeks to evaluate the rapidity of return to base-line levels.

All animals were weighed twice weekly and the amounts of the various extracts given subcutaneously were adjusted accordingly.

TABLE I. AVERAGE MARROW GRANULOCYTIC AND ERYTHROCYTIC ELEMENTS EXPRESSED AS PER CENT OF ALL NUCLEATED CELLS

GROUPS	TOTAL GRANULOCYTIC	TOTAL ERYTHROCYTIC	RUBRIBLASTS	PRORUBRICYTES	RUBRICYTES	METARUBRICYTES
PFPE—ether soluble	42.7	46.5	1.7	3.9	11.6	29.1
PFPE—ether insoluble	59.3	18.3	0.2	0.8	3.7	13.7
Whole plasma—ether soluble	39.9	45.8	1.3	2.3	8.3	33.4
PFPE—postplasma ether extraction	61.1	16.6	0.4	1.1	2.1	13.1
PFPE	43.8	46.0	1.3	2.6	9.4	32.2
Ringer's solution	62.0	15.5	0.4	0.2	1.9	12.5

RESULTS

Erythropoietic stimulation (Figs. 1 and 2, Table I) was evident in the groups injected with the plain PFPE, PFPE-ether soluble, and whole plasma-ether soluble fractions. There was no evidence of accelerated erythropoiesis in the rats given the other plasma extracts or Ringer's solution. As noted before[1-3] with boiled plasma extracts, the erythropoietic stimulation was not accompanied by significant changes in the hemoglobins or hematocrits (Tables II and III). The mean corpuscular volume and mean corpuscular hemoglobin decreased in the rats exhibiting the erythrocytosis and reticulocytosis and the mean corpuscular hemoglobin concentration remained unchanged. Microcytes were evident on the stained films and in the hemacytometer. The increase in marrow cellularity in the animals with the peripheral evidence of erythropoietic stimulation was due to erythrocytic elements involving all red cell precursors. The erythrocytosis and reticulocytosis rapidly disappeared after the injections were stopped.

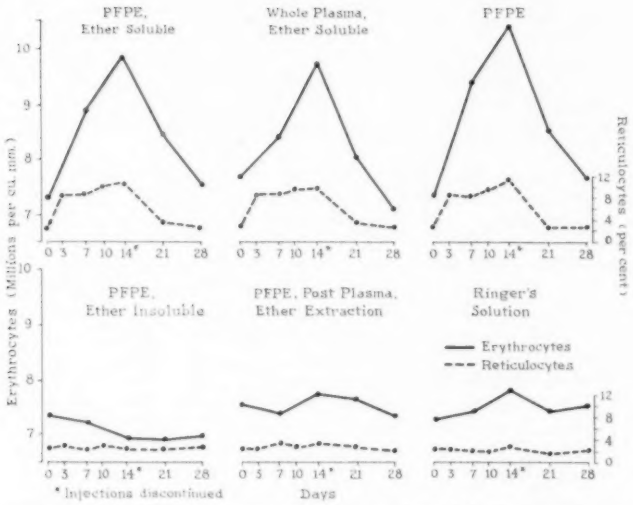

Fig. 1.—Erythrocytosis and reticulocytosis in rats given the ether-soluble fractions of a protein-free plasma extract (PFPE) and whole plasma from anemic rabbits. Average counts of 6 animals injected with each of the above materials.

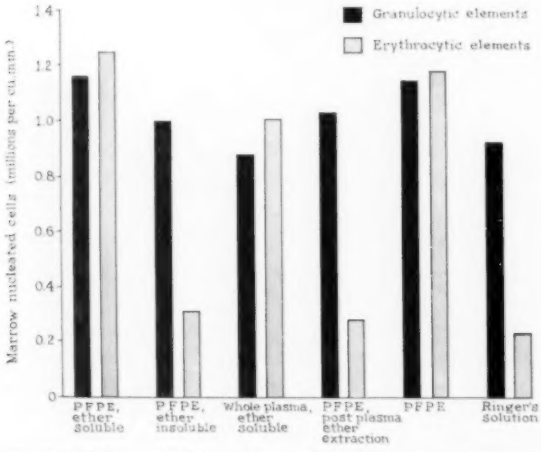

Fig. 2.—Erythrocytic myeloid hyperplasia in rats receiving the ether-soluble portions of a protein-free plasma extract (PFPE) and whole plasma. Marrow nucleated cell counts of animals injected with the ether-insoluble fraction of the former and the latter plasma processed after ether extraction are similar to the controls.

TABLE II. AVERAGE HEMOGLOBIN LEVELS (GRAMS PER CENT)

GROUPS	BASE LINE	1 WEEK	2 WEEKS*	3 WEEKS	4 WEEKS
PFPE—ether soluble	15.2	15.1	14.0	13.8	14.2
PFPE—ether insoluble	15.2	15.1	14.8	14.2	14.7
Whole plasma—ether soluble	14.9	15.2	14.9	14.5	13.9
PFPE—postplasma ether extraction	14.5	15.5	14.6	14.9	14.6
PFPE	14.9	15.2	15.1	14.5	14.8
Ringer's solution	16.1	15.4	15.3	15.0	15.0

*Injections discontinued.

TABLE III. AVERAGE HEMATOCRIT DETERMINATIONS (VOLUMES PER CENT)

GROUPS	BASE LINE	1 WEEK	2 WEEKS*	3 WEEKS	4 WEEKS
PFPE—ether soluble	44.5	43.5	40.9	40.3	40.0
PFPE—ether insoluble	42.9	43.1	42.1	42.1	42.6
Whole plasma—ether soluble	44.2	44.9	45.2	42.5	39.1
PFPE—postplasma ether extraction	44.2	44.3	44.2	42.4	41.6
PFPE	47.0	46.9	45.9	41.7	43.1
Ringer's solution	46.4	45.9	45.6	45.1	45.1

*Injections discontinued.

All animals remained healthy throughout the experimental period and demonstrated no adverse effects from the injections.

DISCUSSION

The observations described above confirm our preliminary studies which indicated that at least one plasma erythropoietic factor which we were studying is soluble in ether. The presence of stimulatory activity in the material extracted twice with ether, with evaporation to dryness interposed between the two extractions, and the absence of such activity in the other fractions obtained from this procedure are findings of considerable import in this regard. They eliminate the possibility of this factor being water soluble and ether insoluble but present at the water-ether interface or carried over in the small amount of water present in the ether phase following extraction. The removal of all activity by ether from previously unmodified "anemic" plasma with identical responses in recipient animals to the ether extract and the boiled extracts affords additional experimental support to the ether solubility of this factor. It also indicates that boiling and precipitation with perchloric acid do not alter the factor as measured by the response in the normal rat.

In the past few years the renewed interest of many investigators in the humoral control of erythropoiesis has led to great advances in our knowledge of the mechanisms that govern red blood cell production. It may be pertinent at this time to review briefly the often strikingly variable experimental results that have been obtained. The original demonstration of erythropoietic stimulatory activity in the serum of rabbits rendered anemic by bleeding was by Carnot and Deflandre in 1906.[4] Confirmation of this finding and the first use

of phenylhydrazine induced "anemic" rabbit plasma as a source of the factor were reported by Gibelli in 1911.[5] Following these observations there appeared scattered reports both in accord[6-8] and in disagreement.[9, 10] The necessity of giving large quantities of "anemic" plasma to produce erythropoietic stimulation was pointed out by Erslev[11] and most likely explains the negative results noted prior to this time. The finding of stimulatory activity in boiled extracts of "anemic" plasma by Borsook and associates[12] and Gordon and his co-workers[13] greatly facilitated the investigation of the plasma erythropoietic factor. Although this observation has since been confirmed by many workers,[1-3, 14-21] other investigators have failed to demonstrate activity in such materials.[22, 23]

The response in the recipient animal to the administration of active plasmas and plasma extracts has also varied. The microcytosis, which we have repeatedly found in the normal rat of Wistar subline injected with boiled plasma extracts, has not been noted by others who have reported increases in hemoglobin and/or hematocrit.[12-18, 20, 21] Gordon and associates[17] have described responses in the rat similar to ours, and Bonsdorff[24] has reported a lack of parallelism between the increase in the erythrocyte count and the other erythroid determinations.

Observations on the possible nature of the humoral erythropoietic factor have also been at considerable variance. The difference of opinion regarding the presence of a stimulating factor in boiled plasma extracts has already been mentioned. The adverse affect of oxygen on activity of the factor[7, 14, 25-27] and its passage through a semipermeable membrane[14, 23, 25, 28] have also lacked uniform confirmation. Erslev and Lavietes[28] concluded that the active factor is attached to, or behaves like, a serum albumin, alpha globulin, or beta globulin. Tohá and his co-workers[14] have suggested that it is possibly a fatty acid ester and not an amino acid, purine, or sugar. In 1949, Sandler[29] isolated batyl alcohol from yellow bone marrow of cattle and demonstrated that the compound had erythropoietic stimulatory activity. Although the source of this material was not plasma, it should be included in a discussion of the humoral control of erythropoiesis. Others have suggested that the factor is a trioxomoloalcoholic sterol[30] or, more recently, a mucoprotein.[21]

Possible explanations for the widely varying views on the nature of the material and its effects are: source and recipient species differences, different methods of demonstrating erythropoietic stimulation, alterations in the active factor by slightly varied techniques utilized in preparing the extracts thus producing a different type of erythropoietic stimulus, and the existence of more than one factor.

The sources of the erythropoietic factor in addition to rabbits and rats, have included monkeys,[31] sheep,[32] dogs,[5] guinea pigs,[5, 6] cattle,[29] and man.[1, 18, 26, 27, 33-37] Erythropoietic stimulatory activity has been demonstrated in plasma, serum, milk,[38] yellow bone marrow,[29] and urine[18, 25] but not in other organs or tissues.[17] The most common experimental methods employed to stimulate its production have been repeated bleedings, the administration of phenylhydrazine, or simulated high altitude.[7, 39, 40] Cobalt[41, 42] and anemia secondary to

total body x-irradiation[3] have also enhanced the donors' plasma erythropoietic activity. Plasmas from the normal sheep fetus,[32] normal pregnant rats,[43] and bled hypophysectomized rats[44, 45] have been shown to be active.

In man erythropoietic stimulatory activity has been demonstrated in the plasma of patients with polycythemia vera and secondary polycythemia[1, 34]; with anemias of varied etiology including pernicious anemia, acute and chronic hemorrhagic anemia, hereditary leptocytosis, sicklemia, leukemia, Hodgkin's disease, and hypoplastic anemia[18, 33, 36, 37]; after venous obstruction to a limb[24]; and in umbilical cord blood of the newborn.[26, 27, 35] Although assays of normal animal and human plasmas have failed to yield definitely positive results, recent studies of concentrated human plasma[36, 37] have shown that activity is present normally but in low concentration.

Rabbits and rats have been the usual recipients in the assay for the factor but dogs and guinea pigs,[5] monkeys,[31] mice,[6, 20] and man[24, 29, 33] have also been employed. The observations made with a wide variety of sources and recipients of the factor indicate a lack of species specificity.

The methods used to demonstrate erythropoietic activity have included, in addition to the determination of the peripheral erythroid values, reticulocyte response, and bone marrow studies; iron[59] incorporation in hemoglobin of normal,[46] hypophysectomized or starved,[37, 41, 42, 45, 47] irradiated,[23] and nitrogen mustard-treated rats[36]; circulating red cell mass in normal[19] and hypophysectomized or newborn rats[34, 43]; and the uptake and disappearance of iron[59] in the plasma, bone marrow, erythrocytes, liver, and spleen[21] and the incorporation of radioactive phosphorus into DNA of bone marrow and spleen of normal rats.[48] Other specially conditioned recipients such as benzene-treated rats[29] and rabbits rendered anemic by bleeding[14] have also been used. The many means of demonstrating activity often make a critical comparison of varying results difficult and undoubtedly explain in large part the conflicting experimental findings previously discussed.

Our studies[36] indicate the existence of at least two plasma erythropoietic factors. One is heat stable, ether soluble, and most likely a lipid (the type assayed in the experiments reported herein). As previously hypothesized, this factor appears to increase the rate of cellular division of already existent marrow erythrocytic precursors with resultant erythrocytosis due to the production of microcytes with shortened survival times.[49] The heat-stable, ether-soluble factor does not accelerate iron[59] incorporation in hemoglobin and does not increase the total circulating hemoglobin or red cell mass. The other factor, which is relatively thermolabile, ether insoluble, and probably protein in nature, augments iron incorporation and hemoglobin synthesis. This effect is demonstrable in unmodified "anemic" plasma and after boiling for 10 minutes but is absent in extracts boiled for 30 minutes or longer.

The concept of there being more than one humoral factor capable of stimulating erythropoiesis is not a new one. In 1938, Tei[8] described in the plasma of bled and phenylhydrazine-treated rabbits an erythropoietic factor that resisted boiling, was ether, acetone, and alcohol soluble, and postulated that it was

probably a lipid. He reported another factor in the plasma of animals subjected to reduced oxygen tension or fed garlic that was thermolabile, ether insoluble, and probably a globulin. More recently, Gley[50] has also described two such factors in "anemic" serum. He believes one to be a trioxomoloalcoholic sterol and the other to have properties closer to the protein group.

In view of the apparent differences in the nature and properties of these two factors, the experimental results would be expected to vary depending upon the method employed to demonstrate activity and the type of material studied. There remains for consideration the possibility of alterations in a single factor produced by slightly different methods of processing the plasmas and plasma extracts for testing. Such changes could conceivably result in a different type of erythropoietic stimulus. Our observations of identical responses in normal rats to ether extracts of whole "anemic" plasma, boiled and perchloric acid precipitated plasma fractions, and ether extracts of the latter indicate that boiling and treatment with perchloric acid do not alter the activity. Studies now in progress concerning the presence of microcytes with abnormal osmotic behavior in polycythemic patients similar to those in normal rats given boiled "anemic" plasma extracts offer additional evidence against the possibility of denaturation of a single factor by processing procedures. Therefore, the differences in response between unmodified "anemic" plasma and extracts prepared by boiling for varying periods of time are, in our opinion, most likely due to the selective removal by boiling of the relatively thermolabile factor, and the apparent disparity in experimental findings described above is the result of the presence of two plasma erythropoietic factors which differ in physical, chemical, and physiologic characteristics.

The precise roles of the plasma factors in normal and abnormal erythropoiesis cannot as yet be defined. The demonstration of activity in concentrates of normal human plasma supports the concept that the humoral factors exert control over the maintenance of normal erythrocytic equilibrium. The enhanced plasma erythropoietic stimulatory activity in experimental situations and in patients with certain anemias and secondary polycythemia is most likely the result of general or local tissue hypoxia. In polycythemia vera evidence suggests that the humoral factors are of pathogenetic importance.[36] With further study of human "anemic" plasmas it may be possible to classify some anemias on the basis of impaired production or defective utilization of the plasma factors.[18, 36, 37, 51] Although the sites of production of the erythropoietic factors are not known, they are apparently not formed in hemopoietic or other radiation sensitive tissue.[3, 28] Recent studies by Jacobson and his associates[41, 42] have implicated the kidney as a possible locus of formation.

There remains a great deal to be learned concerning the mechanisms governing erythropoiesis. Investigative approaches to the multiple aspects of the problem are now under way in many laboratories and much information should soon be forthcoming.

SUMMARY

1. The erythropoiesis stimulating factor in boiled "anemic" plasma extracts is ether soluble.

2. When given to the normal rat, this factor produces erythrocytosis due to microcytes, reticulocytosis, and myeloid erythrocytic hyperplasia. There are no associated increases in hemoglobin or hematocrit values.

3. Ether extracts of whole unmodified "anemic" plasma evoke an identical response in the recipient.

4. Current data indicate the existence of more than one plasma factor. One is heat stable, ether soluble, and most likely a lipid. It stimulates erythroblastic division but not hemoglobin synthesis. Another is relatively thermolabile, insoluble in ether, and probably protein in nature. It appears to augment hemoglobin production.

5. The conflicting experimental findings that have been reported by different investigators studying the humoral regulation of erythropoiesis are reviewed. It is suggested that these apparent discrepancies are due to the presence of two plasma erythropoietic factors which differ in nature and mode of action.

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BILE AND VITAMIN B_{12} ABSORPTION

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THE daily intake of vitamin B_{12} from ordinary dietary sources by an adult man has been estimated to be on the order of a few micrograms, of which 1 or 2 mcg. might be absorbed. The amount of microbial activity of vitamin B_{12} excreted in the urine accounts for only a few millimicrograms of this vitamin. The discrepancy between the amounts of absorbed and excreted vitamin B_{12} may be due to alterations of the molecules into a microbiologically inactive form, or accumulation by the tissues, or excretion by yet another route, not recognized. Available data provide no evidence to support the first hypothesis, and it seems unlikely that the body would continue to retain the absorbed vitamin B_{12}. Such accumulation could lead to the retention of many milligrams of B_{12} in the body, over the period of decades. This is not known to be the case. It is, therefore, of interest to explore the third possibility of some other route of excretion to account for paradoxical vitamin B_{12} imbalance.

One of the important functions of liver is excretory in a manner similar to the kidney. A variety of chemical substances including micronutrients are excreted into the bile. In man, the total volume of bile excreted daily has been estimated to be between 500 and 1,300 ml. per day. If absorbed vitamin B_{12} were eliminated through bile, it would appear in feces. Such phenomenon, if it exists, can explain the anomaly in the difference in excretion. Experiments were conducted to ascertain whether bile plays a role in the absorption of vitamin B_{12}. The study now reported in this communication deals with excretion of subcutaneously administered radioactive vitamin B_{12} in feces probably by way of hepatic bile, and some of the characteristics of B_{12} binding substances in bile.

MATERIALS AND METHODS

Experimental Materials.—

Vitamin B_{12}: Co^{60}-labeled vitamin B_{12} with a specific activity of approximately 1 μc per microgram was employed throughout the study.

Bile: Unless otherwise stated, bile of swine origin was used. It was obtained by pooling the content of 20 gall bladders of pigs shortly after slaughter. Human gall bladder bile was harvested from operatively removed gall bladders of patients with bile duct stone. The source of human hepatic bile was from a patient with bile duct drainage. In preliminary studies desiccated ox bile (processed by Parke, Davis and Co.) was dissolved in distilled water and reconstituted to the original volume of bile.

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Methods.—

The measurement of radioactivity in feces and in urine: Adult rats of the McCoilum strain raised in our own colony were used in the experiments to be reported. Some rats were offered a ration of the basal vitamin-free casein diet to which thyroid powder was added (2 Gm. of thyroid powder per kilogram of diet). They were also employed to study the effect of thyroid on B_{12} excretions.

For the measurement of excretion of radioactivity in urine and feces, the animals were placed in individual metabolism cages. One or two doses of radioactive vitamin B_{12} (20 or 40 mmcg. per dose) were given subcutaneously to the rats. The feces and urine specimens were collected separately, over designated periods (in days). Feces were softened first with distilled water overnight and finally homogenized with concentrated sulfuric acid. Urine and cage washings were evaporated on a steam bath. Both fecal and urinary samples were then brought to 50 ml. and their radioactivity was measured in a well-type scintillation counter. Under the conditions of our studies 1.0 mmcg. of radioactive vitamin B_{12} yielded 120 c.p.m. above the background count.

Choledochectomy: Laparotomy was performed under ether anesthesia, the common bile duct was ligated at two points a little apart, and was severed between the knots in order to secure the complete disconnection of the bile duct from the intestine. The control animals were sham-operated under similar conditions.

The technique of starch electrophoresis[1] and the method of determination of B_{12} binding capacity[2] by dialysis or by microbial adsorption have been described elsewhere. The molecular weight of bile-B_{12} complex was estimated by the procedure of Northrop and associates.[3]

Determination of vitamin B_{12} activity in bile: The presence of inhibitory substance in bile makes it essential to predigest the specimen with an enzyme. The following procedure was found to yield quantitative results. Two milliliters of bile were diluted with an equal volume of distilled water. To this solution was added 1 ml. of 0.5 per cent crude papain solution, and the mixture was incubated for 20 to 40 hours at 37° C. under toluene. The digestion mixture was then acidified with HCl to pH 1.5 to 1.8 and bile pigment was removed by centrifugation. The supernatant fluid was neutralized and diluted to the suitable range for microbial assay by the titrimetric procedure with Skegg's medium.[4] The recovery of added radioactive B_{12} was 90 to 93 per cent after 40 hours of digestion, but only 70 to 75 per cent after 20 hours of digestion.

The above procedure will not be satisfactory for the determination of B_{12} unless the removal of bile pigment is essentially complete. In some instances, particularly for removal of the pigment in hepatic bile, the following additional step was undertaken. Three and one-half per cent aqueous solution of purified human albumin free of vitamin B_{12} was added to bile, the mixture was incubated at 37° C. for an hour, heated at 100° C. for 10

TABLE I. ELIMINATION OF PARENTERALLY ADMINISTERED RADIOACTIVE VITAMIN B_{12} BY RAT

GROUP (NO. OF RATS; AVERAGE WEIGHT)	SPECIMENS COLLECTED (DAYS AFTER FIRST Co^{60}-B_{12} INJECTION)	RADIOACTIVITY RECOVERED (CALCULATED AS B_{12}) IN URINE mmcg. (%)	IN FECES mmcg. (%)	TOTAL EXCRETION IN URINE AND FECES mmcg. (%)
Control	0-6	3.29 (8.12)	6.57 (16.4)	9.86 (24.6)
(5; 285	6-13	0.99	4.12	5.11
grams)	13-21	0.71	2.01	2.72
	21-30	0.87	1.88 ± 0.07*	2.75 (6.8)
	Total in 30 days	5.86 (14.6)	14.58 (36.5)	20.44 (51.1)
Thyroid	0-6	3.33	6.66	9.99
treated†	6-13	1.28	3.95	5.23
(6; 274	13-21	0.85	2.28	3.13
grams	21-30	1.24	2.50 ± 0.11*	3.74
	Total in 30 days	6.60 (16.5)	15.39 (38.5)	21.99 (55.0)

Male rats were used; they received 40 mmcg. Co^{60}-B_{12} by 2 subcutaneous injections (20 mmcg./day).

*Significant at 0.01 level.

†Fed 0.2 per cent desiccated thyroid in ration.

minutes at pH 4.6, and the supernatant fluid relatively free of pigment was obtained for assay. The alkali stability of vitamin B_{12} was measured by the procedure of Hoffmann and associates.[5]

RESULTS

Comparison of the Urinary and the Fecal Excretion.—In the first experiment, two daily doses of 20 mmeg. of radioactive B_{12} were given in two successive days to 11 young adult male rats. Six of them were offered 0.2 per cent desiccated thyroid supplemented ration on the first day of tracer dose administration, while the remaining ones were maintained on the basal diet. Data in Table I demonstrate that radioactivity excreted in the urine of control rats during the 30 days was only 5.86 mmeg. or 14.6 per cent of the test dose, most of which appeared in the urine at the very beginning. In contrast, the fecal excretion during the same period was 14.58 mmeg. or 36.5 per cent, or was more than twice that excreted in the urine. The total radioactivity in both excreta accounted for one-half of the original dose of radioactive B_{12} administered; the remaining portion was undoubtedly retained in the carcass. In the case of

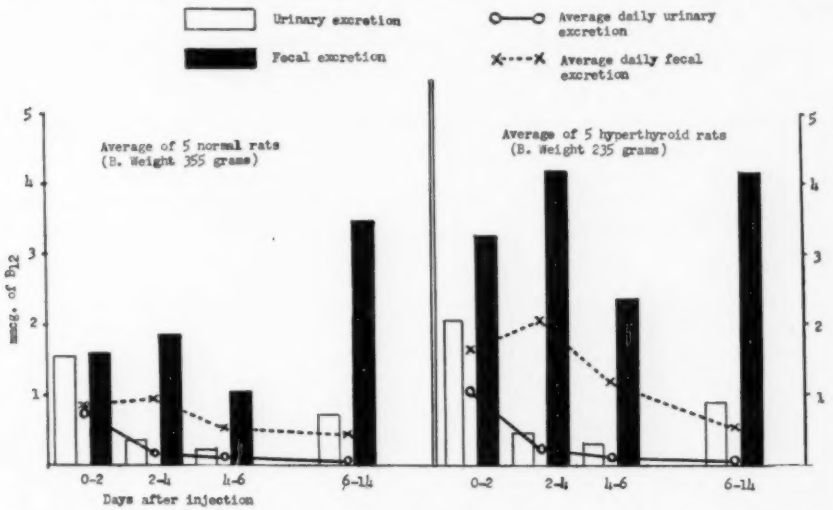

Fig. 1.—Excretion of parenterally administered radioactive vitamin B_{12}. (Following a single subcutaneous injection of 40 mmcg. of B_{12}.)

thyroid-fed animals, the amount of B_{12} excreted both in urine and feces was almost the same as the controls up to 6 days, after which the excretion was increased, so that the fecal excretion between 21 and 30 days was significantly higher in the thyroid-treated group than the control ($p < 0.01$). The total excretion in this group in 30 days was 22 mmeg. or 55 per cent of the administered dose. Since the washings of the cage bottom are not completely free of pulverized feces fragments, the urinary excretion should have been even less than these figures.

In the second study, two groups of 10 male albino rats each were used. Five of them had been fed 0.2 per cent desiccated thyroid for 3 weeks prior to the

test, while the remaining 5 received only the basal diet. All animals were injected subcutaneously with 40 mmcg. radioactive B_{12} in one dose. Bar gram (Fig. 1) shows not only the presence of a large amount of radioactivity in feces, but the fecal excretion in the hyperthyroid animals was almost double that in the controls during the first week. The excretion curve drawn by connecting mean values of the daily excretion demonstrates that the urinary excretion took place shortly after the parenteral administration of radioactive B_{12}, whereas fecal excretion reached its peak several days later.

The discrepancy of the results of the fecal excretion of the thyroid-treated animals in the first and second experiments was probably due to the delayed effect of thyroid. The animals in the second experiment were presumably hyperthyrodic at the time of test while those in the first experiment were likely not so.

Excretion of Radioactive B_{12} in Choledochectomized Rats.—Eight male adult rats were operated on according to the procedure described. The bile ducts of 4 rats were ligated and severed. Since the peak of the fecal excretion following a parenteral dose of B_{12} was found to occur several days after oral feeding, 20 mmcg. of radioactive B_{12} was administered twice on the fourth and fifth days prior to the operation. The urine and fecal specimens were collected two days prior to the operation, i.e., 3 days after the first dose. The results as shown in Table II demonstrate that the decrease in the fecal excretion was statistically significant, being 4.83 ± 0.18 mmcg. in 13 days for the thyroid-fed and 5.96 ± 0.41 mmcg. of the control groups. Jaundice and acholic feces were noted in experimental rats several days following the operation. Necropsy performed on the 26th day of operation revealed a large cystic choledochectasia inside the liver.

TABLE II. EXCRETION OF RADIOACTIVE VITAMIN B_{12} IN RATS WITH LIGATED BILE DUCT

GROUP	NO. OF RATS	AVERAGE BODY WEIGHT (GM.)	2 DAYS PRIOR TO OPERATION FECES (mmcg.)	0-3 DAYS AFTER OPERATION		3-6 DAYS AFTER OPERATION		6-13 DAYS FECES	13-DAY TOTAL FECES
				FECES	URINE	FECES	URINE		
Control	4	386	0.99	1.56	0.45	2.05	0.49	2.38	5.96 ± 0.41
Operated*	4	398	0.88	1.14	0.56	1.61	0.56	2.08	4.83 ± 0.18

Figures represent amounts of recovered Co^{60} as calculated in mmcg. of radioactive B_{12}. Twenty mmcg. radioactive B_{12} was given twice, 5 days and 4 days prior to operation.

*Bile duct was ligated and severed.

TABLE III. EXCRETION OF A SINGLE PARENTERAL DOSE OF RADIOACTIVE VITAMIN B_{12} IN RATS

GROUP	NO. OF RATS	AVERAGE BODY WEIGHT (GM.)	0-2 DAYS		2-4 DAYS		4-6 DAYS		6-DAY TOTAL	
			URINE	FECES	URINE	FECES	URINE	FECES	URINE	FECES
Control	2	330	0.93	0.75	0.17	1.17	0.08	1.91	1.18	3.83 ± 0.44
Operated*	2	356	0.98	0.28	0.20	0.42	0.19	0.62	1.47	1.32 ± 0.31

Figures represent amounts of recovered Co^{60} as calculated in mmcg. of radioactive B_{12}. Radioactive B_{12}, 20 mmcg. as a single dose, was injected subcutaneously immediately after operation.

*Bile duct was ligated and severed.

In another experiment, a single dose of 20 mmcg. radioactive B_{12} was injected immediately after, instead of before, operation, and the specimens were collected in the similar manner. As shown in Table III, the fecal excretion rate was markedly reduced in the choledochectomized animals in the first 6 days, 1.32 ± 0.31 mmcg. against 3.83 ± 0.44 mmcg. of the control.

The two experiments were different with respect to the time of radioactive B_{12} administration, relative to the operation. It was noted that cessation of biliary secretion into the intestine resulted in a marked decrease of fecal excretion in the period shortly after the operation; later in the postoperative stage, however, the fecal excretion rate was not greatly affected by the absence of biliary secretion. It would, therefore, be presumed that the intestinal wall itself excretes a considerable portion of the normal excreta, probably in a later stage following parenteral administration of B_{12}; the bile, on the other hand, excretes B_{12} shortly after the parenteral dose.

Flushing Effect of Large Doses of Unlabeled Vitamin B_{12} on the Fecal Excretion of Radioactive B_{12}.—Since the foregoing experiments demonstrated definitely that a large amount of parenterally administered radioactive B_{12} is excreted in the feces, part of which is probably secreted from the intestinal wall, it is of interest to study whether or not the oral and parenteral administration of large amounts of unlabeled B_{12} can influence the excretion of the parenterally administered radioactive B_{12}.

TABLE IV. FLUSHING DOSE AND FECAL EXCRETION OF INJECTED RADIOACTIVE B_{12} IN RATS

STUDY	GROUP	NO. OF RATS	AVERAGE BODY WEIGHT (GM.)	0-2 DAYS (mmcg.)	2-7 DAYS (mmcg.)	7-DAY TOTAL (mmcg.)
I						
A	Control	3	234	1.35	3.24	4.59 ± 0.46
B	Oral-flush*	3	205	1.22	4.93	6.15 ± 0.75
C	Parenteral-flush†	3	221	1.77	2.86	4.63 ± 0.67

STUDY	GROUP	NO. OF RATS	AVERAGE BODY WEIGHT	0-5 DAYS	5-12 DAYS	12-DAY TOTAL
II						
A	Control	3	214	3.52	3.48	7.00 ± 0.52
B	Oral-flush*	3	236	4.28	3.32	7.60 ± 0.61

Single dose of 40 mmcg. radioactive B_{12} was injected subcutaneously.
*Five hundred micrograms of unlabeled B_{12} was added to 1 Kg. ration.
†Two micrograms of unlabeled B_{12} was injected every day, the first injection being given 6 hours after the tracer dose.

In the first experiment (Table IV, I) 9 rats were divided into 3 groups; the control (A), the oral-flush group (B) receiving crystalline B_{12} added to the ration at the level of 500 mcg. per Kg., and the parenteral-flush group (C) receiving 2 mcg. B_{12} daily by injection. The fecal excretion in 7 days following a single injection of 40 mcg. radioactive B_{12} was 4.59 ± 0.46, 6.15 ± 0.75, and 4.67 ± 0.67 mmcg. for groups A, B, and C, respectively. The difference between the control and groups B or C was not statistically significant.

In a separate experiment (Table IV, II), only groups B and A were compared; the fecal collection was continued for 12 days, however. Even then the difference between these two groups was not statisticaly significant.

These data indicate that the fecal or intestinal excretion of radioactive B_{12} is not significantly affected by either oral or parenteral flushing. The postulated intestinal excretion of vitamin B_{12}, therefore, is not simply a matter of exchange of this vitamin between the intestinal mucosa and the intestinal content. Neither does this pattern of excretion seem to be inhibited by the presence of a large quantity of B_{12} in the intestinal lumen which is expected to be absorbed by passing through mucosa.

Vitamin B_{12} Activity in Bile.—Bile specimens of swine and human origins were assayed for microbiological activity of vitamin B_{12} before and after digestion with papain. To another aliquot of biological specimens was added radioactive vitamin B_{12} to contain 10 mmcg. of B_{12} per ml. of bile solution. Microbiological activity, and, in the case of radioactivity-containing bile, radioactivity were measured. The following points are of interest. In the first place, only 0.8 of mmcg. of B_{12} activity was found in the undigested bile. The activity in the swine bile solution was increased from 4.5 to 8.8 mcg., thus indicating the liberation of B_{12} activity due to digestion. To test that the increase in B_{12} activity after incubation is related to papain digestion, a portion of the bile-papain mixture was heated at 70° C. to destroy the enzyme which cleaves the union between B_{12} and the binding substance. The B_{12} activity in the preheated samples was not increased on incubation. Determination of radioactivity in the various aliquots shows that B_{12} can be liberated and quantitatively recovered upon digestion for 40 hours, but only partially after 20 hours. It is also interesting to note that papain digestion did not increase B_{12} in the bile of human gall bladder, presumably due to a much smaller portion of the binding substance.

DISCUSSION

The daily intake of vitamin B_{12} from ordinary dietary sources by an adult man is many times larger than that excreted in the urine. Data presented in this communication suggest that another route of excretion must be recognized and support the concept that vitamin B_{12} may be bound to chemical substances in bile and finally excreted in the feces. Suggestive of this possibility is the appearance of injected radioactive vitamin B_{12} in feces, in amounts several times larger than that present in the urine.

It is interesting to note that the bile fluid has a great capacity to bind B_{12}. This binding substance is apparently chemically distinct from that in intrinsic factor preparations. The union of vitamin B_{12} with the latter can be dissociated upon heating at 100° C., whereas, the B_{12}-bile complex is stable at this temperature. The binding substance in bile is relatively free from protein and has a large molecular weight and contains a large percentage of glucosamine.

Our data thus throw additional light on the mechanism of the absorption of vitamin B_{12}, and are pertinent to interpretation in the measurement of absorption of orally fed vitamin B_{12} by the fecal excretion method of Heinle and

associates.[6] The latter procedure has been found to yield excellent results. It is obvious from this study that fecal radioactivity does not represent exclusively the unabsorbed B_{12} as is now commonly assumed.

SUMMARY

The injection of a small but physiologic dose of radioactive vitamin B_{12} to normal, thyroid-fed, or laparotomized rats results in the appearance of radioactive vitamin B_{12} in feces. It is postulated that the absorbed B_{12} is bound to bile and excreted in the feces. In addition, the intestinal wall itself must play an important role in the excretion of vitamin B_{12}.

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IRON ABSORPTION

I. Factors Influencing Absorption

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With the Technical Assistance of B. Loden and A. Melly

WHEN the iron content of the body is altered by some cause, such as blood loss, balance is re-established mainly by changes in the amount of iron absorbed from the gut.[1] Because of its capacity to respond to the needs of the body, the gastrointestinal mucosa has been regarded as the chief regulator of iron balance.[2] The mechanisms by which this control is exerted are, however, still in doubt. One problem has been the technical difficulty in accurately measuring iron absorption. Recently, precise methods employing radioiron have been developed, but only limited observations have been made concerning the influence of anemia, anoxia, plasma iron level, iron stores, and altered erythropoiesis on iron absorption.

The present paper reports the results of studies involving several aspects of iron absorption. Experiments in animals were carried out in a variety of situations and the hypotheses which emerged were tested in man. The investigation was broadened to include observations on absorption of iron salts by normal subjects, by individuals after acute blood loss, and by patients with iron deficiency anemia. Information was obtained on the relative importance of certain internal factors in the regulation of iron absorption and, in addition, a rational approach to the problem of iron therapy was developed.

MATERIALS AND METHODS

Sprague Dawley rats weighing 250 to 350 grams, New Zealand white rabbits weighing 2.5 to 3.5 kilograms, and a strain of adult field mice with hereditary spherocytosis[3] were employed in various studies. In each experiment control animals of the same sex, age, weight, and dietary background were employed. The purpose was to indicate whether a specific factor altered absorption in these animals as compared to control animals. Variations, particularly in the amount of iron administered, made it impractical to interrelate individual studies.

Radioiron was administered directly by gastric intubation. Rats were rendered anemic by removing 5 ml. of blood at 5 and 3 days prior to study, or by the parenteral administration of an ethanolic solution of phenylhydrazine (30 mg./Kg.). Hemolytic anemia was also induced by the prior injection of methyl cellulose intraperitoneally twice

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weekly as described elsewhere.[4] Excess body iron stores were produced in rats, rabbits, and mice by the intramuscular injection of iron-dextran (Imferon*). Exchange transfusion was performed in rats by the insertion of plastic cannulae into the femoral artery and vein with the simultaneous removal of circulating blood and the injection of blood previously obtained from donor animals.[5]

Normal human subjects studied had plasma iron concentrations between 80 and 160 μg/100 ml. plasma and hematocrits between 42 and 48 per cent. In patients the criteria of iron deficiency were plasma iron levels below 50 μg, the absence of marrow hemosiderin, and a response to iron therapy.

Absorption studies were performed on normal volunteer subjects, fasting or after meals, as specified under results in individual experiments. Phlebotomy was also employed in normal subjects to enhance iron absorption. Unless otherwise specified, such subjects were bled 400 ml. on the ninth and seventh days before the absorption study was performed.

Absolute iron absorption was measured by the double isotope method described by Saylor and Finch.[6] In this technique a weighed amount of Fe^{59} either as iron citrate or as iron bound to fresh plasma† was injected intravenously and at the same time a solution of iron (Fe^{55}) was given orally. Standards were then made from both solutions given. Fourteen days after administration of the two isotopes, a blood sample was collected and wet-ashed with sulfuric and perchloric acids. Samples were precipitated with the addition of carrier iron to a total of 10 mg. and electroplated as described previously.[7] Counting for Fe^{55} was done with an argon-filled geiger tube having a beryllium filter, whereas Fe^{59} was determined with a helium-filled tube. The cross-counting for which appropriate correction was made was less than 4 per cent in either direction. Calculation of results:

$$\text{Percentage absorbed} = \text{yl} \frac{\frac{(\text{x})}{(\text{xl})} \times 100}{\text{y}}$$

When yl = activity of Fe^{55} in blood sample (c.p.m.)
y = activity of Fe^{55} given by mouth (c.p.m.)
xl = activity of Fe^{59} in blood sample (c.p.m.)
x = activity of Fe^{59} injected intravenously (c.p.m.)

In several studies iron absorption was computed by measuring stool radioiron content. To determine stool activity, a 10-day stool collection was transferred from waxed cartons to a large weighed beaker. The bulked specimen was then covered with concentrated sulfuric acid for 10 to 14 days with occasinal stirring and addition of acid to a total of about 5,000 ml. Gentle heating was applied when necessary to produce a homogenous mixture. Ten gram aliquots were electroplated in the usual manner prior to counting.[7] Since the validity of methods such as these must ultimately rest on direct comparisons, results of absorption by the stool and double isotope methods in 4 normal subjects are summarized in Table I.

TABLE I. COMPARISON OF STOOL RECOVERY AND DOUBLE ISOTOPE METHODS FOR MEASURING IRON ABSORPTION IN 4 HUMAN SUBJECTS

	STOOL METHOD (PER CENT ABSORPTION)	DOUBLE ISOTOPE METHOD (PER CENT ABSORPTION)
1	5.3	4.8
2	15.2	12.5
3	5.5	2.6
4	20.9	16.6

*Obtained through the courtesy of the Benger Company, England, and Lakeside Laboratories, Milwaukee, Wis.

†It is essential that there be either adequate capacity of the recipient's plasma to bind the injected iron citrate, or that iron be bound to a plasma with adequate binding capacity before its injection.

In certain studies on human volunteers the major interest was centered not so much on the absolute amount of iron absorbed as on the relative amounts absorbed under two different sets of conditions. One isotope of iron in one form was given by mouth on the first morning, while the second was given by the same route on the following morning. The order of administration of the two test doses was alternated in different subjects. Analysis of activity in the blood drawn two to three weeks later permitted a comparison of absorption between the two test doses in the same subject. In order to estimate the absolute absorption, the per cent of red cell utilization of absorbed iron must be predictable. This was assumed to be 90 per cent in normal and iron deficient subjects and the blood volume was estimated at 68 ml./Kg. Estimated per cent absorption then equaled:

$$\frac{\dfrac{\text{activity per ml. blood} \times \text{blood volume (ml.)} \times 100}{0.9}}{\text{total radioactivity administered}}$$

The actual amount of radioactivity given varied in different studies depending upon the anticipated absorption and on the total amount of administered elemental iron. In man, the amount of Fe^{55} varied between 50 and 100 μc when carrier iron of between 100 and 500 mg. was added. $Iron^{59}$, in doses of approximately 1 μc, was given intravenously as ferrous citrate or was bound in vitro to the normal plasma when the patient's plasma iron binding capacity was inadequate.[8] The specific activity of the two isotopes used varied between 1 and 3 $\mu c/\mu g$. With the double isotope method of measuring absorption, the dosage of each isotope was designed to provide nearly equal counts in the circulating blood.

Plasma iron was estimated by the method of Bothwell and Mallet,[9] and of Kitzes, Elvehjem, and Schuette.[10] Bone marrow iron was estimated from sternal aspiration as described by Rath and Finch.[11] Hemoglobin was determined as oxyhemoglobin in an Evelyn colorimeter and hematocrits were spun at 1,800 g for 30 minutes. Reticulocytes were stained with cresyl blue and the number in 1,000 erythrocytes counted.

RESULTS

A. Internal Factors Regulating Iron Absorption.—The object of these studies was to delineate the effect of alterations within the body on iron absorption. In order to minimize the possible interference which local gut factors might have on such absorption, initial studies were done with animals and experimental subjects in a fasting state. Iron was given as a freshly prepared solution of ferrous sulfate. The usual dosage of iron was standardized at 0.25 mg./Kg. of elemental iron in experimental animals. Data obtained may be summarized under four headings.

1. Effect of the rate of erythropoiesis on iron absorption: Increased erythropoiesis was produced in several different ways. One injection of phenylhydrazine was given to rats and absorption was measured one week later when the reticulocyte count was high and the hematocrit was rising rapidly. In rabbits, multiple injections were given and absorption was measured at variable times thereafter. Methyl cellulose injected rats which had increased red cell production and destruction, but little anemia, and mice with increased erythropoiesis due to hereditary spherocytosis were also studied. These results are summarized in Table II and consistently indicated an increase in iron absorption in animals with increased erythropoiesis as compared to normal animals of

the same species given the same dose of iron. Additional studies in rabbits given multiple injections of phenylhydrazine, while again indicating increased absorption with increased erythropoiesis, showed no correlation between a rising or falling hematocrit or with the level of plasma iron.

TABLE II. EFFECTS OF INCREASED ERYTHROPOIESIS ON IRON ABSORPTION

DIAGNOSIS	NUMBER OF ANIMALS	DOSAGE OF IRON (μG)	ABSORPTION (± S.D.) (PER CENT)
Phenylhydrazine in rats	5	50	59 ± 19
Controls	6	50	21 ± 8
Phenylhydrazine in rabbits	8	1,000	30 ± 19
Controls	8	1,000	12 ± 11
"Methyl Cellulose" in rats	6	100	16.2 ± 6.2
Controls	4	100	10.3 ± 1.9
Congenital spherocytosis in mice	7	3.5	7.2 ± 2.2
Controls	7	3.5	4.8 ± 1.9

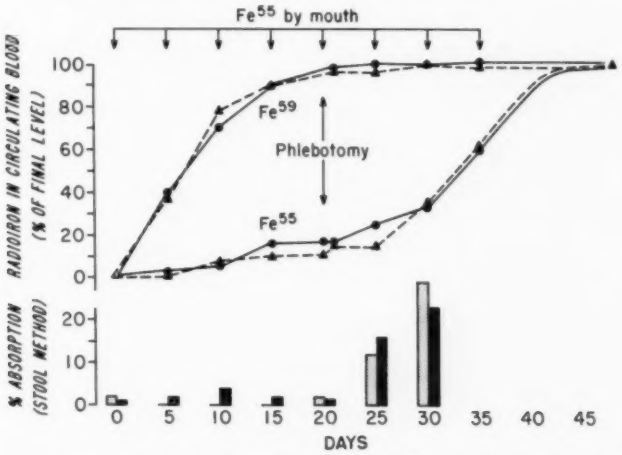

Fig. 1.—Effects of phlebotomy on iron absorption in 2 human subjects as measured by stool recoveries and by circulating radioactivity in the blood. During the control period there is almost complete recovery of orally administered Fe^{59} in stool and little radioactivity in circulation. After phlebotomy both measurements indicate an increased absorption of iron.

In an attempt to clarify further the time relationship between increased absorption and erythropoiesis, studies were carried out in experimental subjects before and after phlebotomy. As shown by stool measurements as well as by blood activities in two subjects, increased absorption occurred within 5 days of phlebotomy (Fig. 1). Absorption was also measured in normal fasting subjects given 25 mg. of iron (Fe^{59} sulfate) and phlebotomized 8 ml./Kg. on the following day. Repeat absorptions were then done with Fe^{55} under identical conditions through the following week and the pre- and postphlebotomy ratio was determined (Table III). In the first four days values were variable, but thereafter absorption showed a moderate but consistent increase.

The effect of *decreased erythropoiesis* was also studied. To retard red cell production, rats were made polycythemic by exchange transfusion with packed erythrocytes. The hemoglobin level after exchange in these animals was above 20 Gm. per cent. As a control, another group of rats was submitted to the same procedure except that they were exchanged-transfused with normal whole blood. Reticulocyte counts were depressed in the polycythemic animals, although the time lag in marrow response made this less conspicuous at two days. Absorption studies (Table IV) showed a reduction in iron absorption by polycythemic animals as compared to controls.

TABLE III. THE ABSORPTION OF A DOSE OF 25 MG. ELEMENTAL IRON BEFORE AND AFTER PHLEBOTOMY OF 8 ML. PER KILOGRAM IN 12 HUMAN VOLUNTEERS

SUBJECT	PLASMA IRON	BASAL ABSORPTION (PER CENT)	DAYS AFTER PHLEBOTOMY	PLASMA IRON	SECOND ABSORPTION (PER CENT)	$\frac{S.A.}{B.A.} \times 100^*$
1	113	12.4	2	140	8.2	66
2	135	12.5	2	128	11.4	91
3	81	9.9	3	72	14.6	147
4	111	13.6	3	92	21.7	160
5	142	7.8	4	64	5.6	72
6	123	8.8	4	96	7.1	81
7	133	13.4	5	50	18.3	137
8	215	9.5	5	94	15.5	163
9	118	17.4	7	71	27.0	155
10	113	12.6	7	83	15.0	119
11	193	5.0	11	103	11.5	230
12	112	9.0	11	76	12.0	133

*$\frac{\text{Second absorption}}{\text{Basal absorption}} \times 100$.

TABLE IV. EFFECT OF DECREASED ERYTHROPOIESIS ON IRON ABSORPTION IN RATS

ANIMALS	DAYS FOLLOWING TRANSFUSION	DOSAGE (μG)	HEMOGLOBIN (GM.)	RETICULOCYTE COUNT (PER CENT)	ABSORPTION (PER CENT)
Control	2	20	14.8	2.8	19.4
			15.5	2.1	25.0
Polycythemic	2	20	20.2	1.1	0.6
			20.6	1.4	2.4
Control	5	100	19.3	1.4	8.0
			16.3	1.5	7.4
			17.8	1.5	6.8
			16.7	0.8	15.8
			16.9	1.8	14.3
Polycythemic	5	100	21.0	0.3	4.7
			19.4	0.1	2.0
			19.6	0.3	4.6

2. Effect of change in body iron stores on absorption of iron: Iron deficiency in rats was produced by feeding a low-iron diet from the time of weaning, and excessive iron stores were created by the injection of 100 mg. of iron 3 months before the study. Absorption in these animals was compared to that in normal animals (Fig. 2). In the group of 6 animals with excessive iron, absorption was decreased to 3.9 ± 0.9 per cent as compared to the normal group of animals with 20 ± 7 per cent absorption. The 4 iron deficient animals showed an increase to 47 ± 13 per cent. A comparable increase in absorption of iron deficient patients as compared to that in normal subjects is shown later.

3. Combined effect of iron stores and erythropoiesis on absorption: The studies previously described indicated that increased erythropoiesis was associated with increased absorption of iron and decreased erythropoiesis with decreased absorption. The interplay of two opposing factors was illustrated by the following experiment. The erythroid marrow was activated by bleeding two groups of animals, one having previously had normal iron stores and the other having been loaded with 100 mg. of iron by parenteral injection one month previously. As compared to the normal absorption of 20 per cent, 5 phlebotomized animals with hematocrits of 40 ± 2 per cent absorbed 40 ± 17 per cent. The

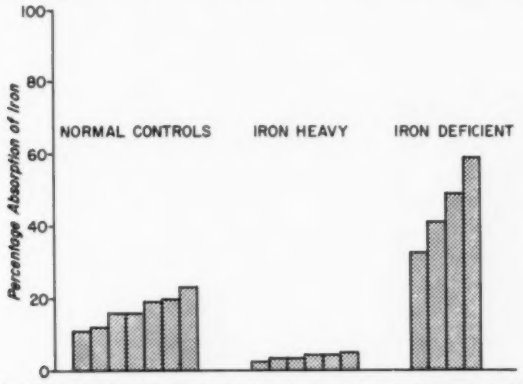

Fig. 2.—Effects of changes in body stores on iron absorption in rats.

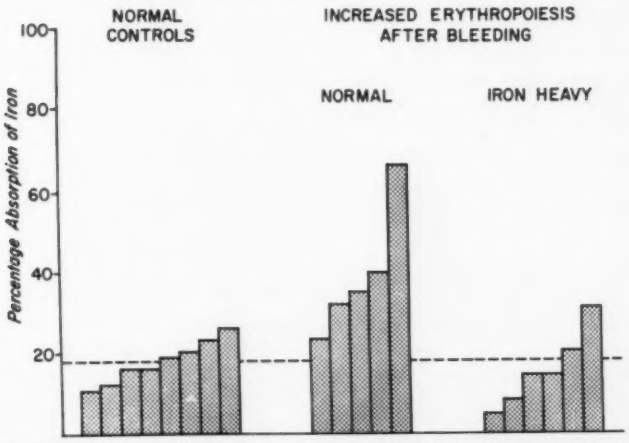

Fig. 3.—The combined effects of increased erythropoiesis and increased stores on iron absorption in rats. As compared with the group of normal animals, increased erythropoiesis increased iron absorption. However, the composite effect of a similar increase of erythropoiesis in animals with excess body iron was a normal absorption.

absorption by the 6 animals who were both iron loaded and phlebotomized was within the normal range (15 ± 9 per cent) (Fig. 3). Thus, with one factor tending to increase iron absorption and another to decrease it, the results fell within the normal range.

4. Effect of the plasma iron level on absorption from the gut: In the studies on normal and phlebotomized subjects there was no evident correlation between plasma iron and the amount of iron absorbed. To obtain more definite information, absorption studies were performed before and after the plasma iron had been raised to saturation of the iron-binding protein in 3 rabbits and in 2 normal subjects who had been previously phlebotomized. This elevation of plasma iron was accomplished by the infusion of erythrocytes rendered nonviable through in vitro storage in sodium citrate for 3 weeks. The marked increase in plasma iron which occurred between 2 and 8 hours after injection of the cells, due to the catabolism of their hemoglobin, did not significantly affect absorption (Table V).

TABLE V. EFFECT OF PLASMA IRON LEVEL ON IRON ABSORPTION

RABBIT STUDIES	Fe DOSAGE	PLASMA IRON (μG/100 ML.)		ABSORPTION (PER CENT)	
		BEFORE	AFTER	BEFORE	AFTER
R-1	500 μg	55	328	53	43
R-2	500 μg	162	314	87	81
R-3	500 μg	114	271	12	5
CLINICAL STUDIES					
S-1	5 mg.	92	315	40	53
S-2	50 mg.	87	267	8	7

B. Effects of Some Local Factors on Iron Absorption.—Many questions which fall outside the scope of this paper arise concerning local factors within the gastrointestinal lumen. However, certain observations relating to the form and amount of administered iron and the effects of food and ascorbic acid are presented because of their practical importance in iron therapy.

TABLE VI. EFFECT OF FORM OF IRON ON ABSORPTION IN PHLEBOTOMIZED NORMAL SUBJECTS

SUBJECT	DOSE OF IRON (MG.)	ABSORPTION (PER CENT)	
		FERROUS SULFATE SOLUTION	"QUICK" RELEASE SPANSULES
1	16	18.8	23.8
2	16	36.8	21.0
3	16	15.0	8.0
		FERROUS SULFATE SOLUTION	"SLOW" RELEASE SPANSULES
4	48	24.0	4.0
5	48	17.4	4.6
6	48	38.0	7.0
		FERROUS SULFATE SOLUTION	CALCIUM IRON CITRATE
7	50	9.9	3.0
8	50	10.7	8.7
9	50	20.8	22.0
10	100	4.7	5.5
11	100	4.5	2.4

1. Form of ingested iron: The absorption of a solution of ferrous sulfate was compared with that of ferrous sulfate in the form of quickly disintegrating

and slowly disintegrating spansules.* The spansule and ferrous sulfate preparations were fed to fasting phlebotomized subjects on alternate mornings. Results (Table VI) indicated little difference between the iron solution and the rapid release spansule, but did show a marked reduction in the amount of iron absorbed from the preparation with a slower release through the lower intestinal tract. Studies comparing ferrous sulfate with iron in the form of a calcium citrate chelate† showed little difference between these two compounds.

2. Relation between dosage of iron and amount absorbed: Data on 6 normal male volunteers, 13 other normal volunteers who had previously been phlebotomized, and on 8 iron deficient patients are summarized in Fig. 4. All tests were done with subjects in a fasting state and all were given freshly prepared solutions of ferrous sulfate varying in dosage from 5 to 500 mg. of iron. Normal subjects showed such individual variations as to obscure the effect of dosage on absorption. However, in phlebotomized and iron deficient subjects, the higher absorption with increases in iron ingestion was better defined. The 3 groups studied appeared to differ largely in the capacity of the mucosa to transport iron. The absorption ratio between the normal, phlebotomized, and iron deficient subjects was approximately 1 : 3 : 9.

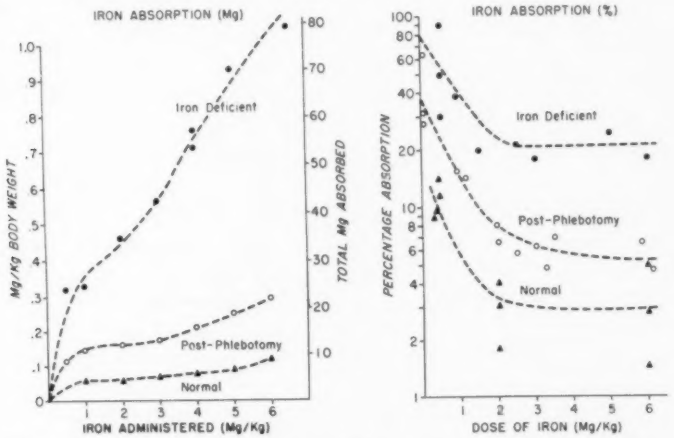

Fig. 4.—The absorption of varying dosages of iron in normal, phlebotomized, and iron deficient subjects.

3. The effect of ascorbic acid and cobalt on iron absorption: In animals given a constant amount of ascorbic acid and increments of oral iron (Fig. 5), it was possible to demonstrate an enhancement in iron absorption. In man, smaller dosages of ascorbic acid, in keeping with amounts feasible for clinical administration, were given. The dosage of ascorbic acid varied from 100 to 1,000 mg., and iron was given in amounts of either 1 or 2 mg./Kg. The subjects were

*These were obtained through the courtesy of Smith, Kline & French Laboratories, Philadelphia, Pa.

†This material, ferrous calcium citrate with tricalcium citrate, distributed as Rarical, was obtained through the courtesy of the Ortho Pharmaceutical Corporation, Raritan, N. J.

phlebotomized prior to absorption tests and studied either fasting or postprandial. The effect of ascorbic acid was not pronounced, as indicated by an average absorption of 12 per cent in all studies as compared with control values of 9 per cent (Table VII).

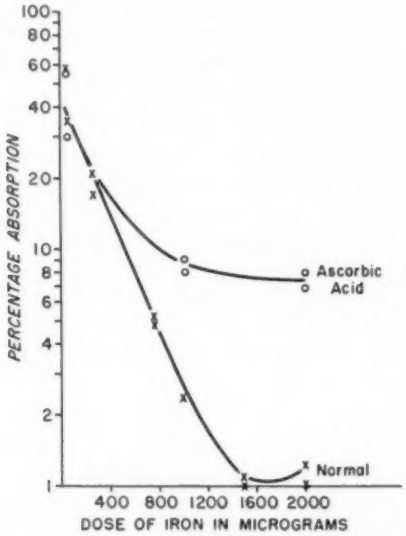

Fig. 5.—The effect of 10 mg. of ascorbic acid given with varying doses of iron was compared with absorption of iron alone.

TABLE VII. EFFECTS OF ASCORBIC ACID ON IRON ABSORPTION IN PHLEBOTOMIZED NORMAL SUBJECTS

METHOD OF Fe ADMINISTRATION	DOSE OF ASCORBIC ACID (MG.)	ABSORPTION (PER CENT)	
		CONTROL	WITH ASCORBIC ACID
Fasting	100	10	11.6
Dose of 1 mg./Kg.	200	17	22.0
	200	8	13.0
	500	6	8.0
	1,000	12	16.0
Fasting	100	6.9	4.9
Dose of 2 mg./Kg.	200	7.3	6.3
	400	3.6	7.4
	600	6.1	7.2
	1,000	8.2	6.1
After Meals	100	8.0	11.0
Dose of 1 mg./Kg.	200	13.0	15.0
	500	18.0	24.0
	1,000	7.0	14.0

The effect on iron absorption of 80 to 120 mg. per day of cobalt given as cobalt chloride was also determined. In two subjects absorption studies were carried out after 7 days and in two others after 14 days of cobalt administration. Absorptions before and after cobalt were 3.7 versus 9.8 per cent, 2.3 versus 5.5 per cent, 9.4 versus 9.2 per cent, and 9.8 versus 8.1 per cent.

DISCUSSION

Ingested iron is absorbed in the ferrous form largely by the duodenum. The absorbed iron passes through the mucosal cells into the blood stream where it is bound by a beta-1 globulin of the plasma. Normally about 90 per cent of this iron is incorporated into the hemoglobin and is found in the circulating red cell mass within 2 or 3 weeks; in iron deficiency anemia absorbed iron may be almost quantitatively recovered from circulating erythrocytes.

The most accurate early information in the literature of iron absorption related to iron deficiency anemia, where the amounts of iron absorbed were great, and absorption could be measured by the increase in circulating hemoglobin. Initial measurements employing radioiron followed the same principle of measuring the radioactivity in the circulating red cell mass.[12] This measurement was found to be unreliable in certain diseases due to a significant and variable distribution of radioactive iron into tissues other than the erythrocyte. This led to the development of a stool recovery method where absorption was determined from the difference between iron administered and that recovered in the feces.[13]

While there is no reason to question the validity of this method on theoretical grounds, since excretion of absorbed iron into the gut is negligible, there are practical difficulties. Not only is it a problem to ensure complete collection over a period of 8 to 12 days, but the per cent absorption is often so small that technical errors are magnified.* At these low levels it is possible to determine absorption more accurately by a double isotope method, whereby the second isotope measures distribution between tissues and red cells. The ratio of the two isotopes then indicates the total amount absorbed without the necessity of a blood volume determination. The reliability of this method at a dose range of 0.5 mg./Kg. iron in measuring isotope distribution between red cells and other tissues has been previously reported.[6] These studies have been extended over a wide dose range up to 10 mg./Kg. with similar results.[16] Combined stool and double isotope measurements on 4 subjects gave an average of 12 per cent and 9 per cent, respectively (Table I). In another subject given radioiron every 5 days over a 42-day period, average daily absorption was 5.4 per cent by the stool method and 3.9 per cent by the double isotope technique (Fig. 1). A further indication of the general reproducibility of the double isotope technique was found in examination of the consistency in repeat measurements in the same individual where the two sets of conditions produced similar absorption (Tables V, VI, and VII).

*In certain conditions, e.g., infections, lymphomas and hemolytic anemias,[13] and rheumatoid arthritis,[14] absorption as judged by stool recoveries has been found sometimes to be a good deal greater than would have been apparent from blood levels alone. Such results would presume that only a small portion of the radioiron entering the plasma is utilized for hemoglobin production in these conditions. In recent years, however, it has been possible, using iron of high specific activity, to tag the plasma iron-binding globulin without exceeding its binding capacity and so find out what proportion of the plasma iron is concerned with red cell production.[15] Under these circumstances, with the exception of certain hemolytic anemias, it has been shown that red cell utilization is only rarely below 50 per cent and that it is usually a good deal higher.[16] These findings suggest the need for re-evaluating some of the earlier results for absorption obtained by stool recovery techniques.

It is apparent that the method employed to measure iron absorption must be modified depending on the problem at hand. When the per cent incorporation of radioiron is 80 to 100 as in the normal or iron deficient subject, a determination of total red cell activity at two weeks will suffice. Comparison can then be made of two absorption tests employing different isotopes. If the per cent utilization of radioiron for red cell production is not predictable, either the double isotope method previously described or the stool recovery method must be employed. While the double isotope method has been preferred for most of the studies reported here because of its simplicity and greater accuracy, this method is not feasible when there is a complete failure of erythropoiesis. Accordingly, in these studies a variety of methods have been employed according to the experimental situation.

A further adjunct to the evaluation of absorption in man has been the use of phlebotomy. This has proved particularly useful in testing iron preparations of unknown value, since the amount of iron absorbed in normal subjects is often too small for definitive results. The absorption by phlebotomized subjects over a range of iron dosage was 2 to 3 times normal (Fig. 4) and appeared somewhat more uniform from subject to subject.

Normal man was taken as a reference point in studies of iron absorption. It seemed important to define normality insofar as possible as it is related to erythropoiesis and body iron content including normal red cell, plasma, and marrow iron values. In such subjects, mean absorption of ferrous salts ranged between 2 and 8 mg. as the dose of iron was increased from 20 to 400 mg. (Fig. 3). One point of interest was the variation in absorption between individual normal subjects. While the explanation for this may lie in changes too subtle to measure, it does emphasize the difficulty of evaluating absorption in the normal subject unless comparative measurements between two compounds are made.

Both the status of iron stores and the level of erythropoiesis have been shown to influence iron absorption. The increased absorption of both iron salts and dietary iron in iron deficiency has been well established.[2, 17] Studies here in animals also indicated depressed iron absorption when iron stores were increased above normal. To what extent this may apply within the narrower limits of expansion and contraction of the normal iron stores is not clear. Available evidence suggests that once the anemia of iron deficiency has been corrected, iron absorption returns to a normal base line despite depleted stores.[18, 19] More data are required on this point.

The second important factor influencing absorption is the activity of erythroid marrow. In the present study, absorption has been shown to be either increased or decreased in direct relationship to the level of erythropoiesis in experimental animals given iron salts. These findings support previous reports of increased absorption related to increased erythropoiesis in experimental animals.[20, 21] In the phlebotomized but otherwise normal subject, absorption has

been shown to be consistently elevated (Fig. 1). The lack of any relationship between iron absorption and anemia per se is indicated by clinical studies in a following report.[22]

In summary, a variety of experiments has provided abundant evidence to indicate that interplay between body iron content and erythropoietic activity affect iron absorption.

The effectiveness of oral iron in treatment of iron deficiency anemia is indicated by comparing the amount of iron which may be absorbed with the needs of the marrow for erythropoiesis. These marrow requirements, estimated at 25 to 50 mg. per day,[23] are met by a single dose of 150 to 200 mg. of iron. Comparative studies of different forms of iron indicate that iron must be in a form available to the upper intestinal tract, and that ascorbic acid* in doses of 100 to 1,000 mg. and cobalt in daily dosage of 80 to 120 mg. over one to two weeks do not offer any significant advantage in iron therapy. A citrate-calcium-chelate of iron was shown to be nearly as well absorbed as ferrous sulfate. While these absorption data have no bearing on the tolerance of various preparations, they are consistent with many observations in the literature that ferrous salts in a form available to the upper intestine are the most suitable forms of medicinal iron, and a daily dosage of 0.2 to 0.3 mg. iron per kilogram, equivalent to 0.2 Gm. exsiccated ferrous sulfate 3 times per day, is usually adequate.

Certain speculations concerning the mechanism of iron absorption seem justified. The role of iron stores and erythropoiesis in modifying absorption has been shown. The time relationship of erythroid marrow and increased iron absorption is of particular interest. Following phlebotomy marrow iron uptake increases within 24 hours,[8] while absorption shows a lag of several days. The connection between marrow and intestinal mucosa is not through the plasma iron or hemoglobin level. Another mechanism whereby the needs of the bone marrow are felt by the mucosa must be operative, although this has not been directly demonstrated.

SUMMARY

Iron absorption has been quantitated by a double isotope method, by stool recovery and by comparative measurements of iron absorption from two compounds as they appeared within the red cell mass. Employing these isotope measuring techniques, the regulation of absorption of small and large doses of iron has been studied.

Both the size of iron stores and the rate of erythropoiesis have been shown to influence the absorption of iron. The larger the iron stores the less iron absorbed whereas the greater the red cell production, the greater the amount of iron absorbed.

Absorption in normal and phlebotomized subjects and in patients with iron deficiency was measured. The effects on absorption of the amount of iron, the form in which it is administered, and of supplements were also evaluated.

*The effect of ascorbic acid would appear different when employed with therapeutic doses of iron salt as compared to its administration with food iron, when it has been shown to have a clear-cut effect in enhancing absorption.[22]

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IRON ABSORPTION

II. The Absorption of Radioiron Administered With a Standard Meal in Man

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IN THE preceding report[1] evidence has been presented that iron absorption is influenced by at least two factors, i.e., the state of the body iron stores and the erythropoietic activity of the marrow. These conclusions were drawn from studies performed in both laboratory animals and human subjects. In an attempt to minimize the numerous variables attendant upon such an investigation, all experiments were done with subjects in the fasting state and soluble iron salts were used. With this general information on iron absorption in hand, it seemed of importance to evaluate the absorption of iron in food by individuals in a healthy state and in patients suffering from any one of a variety of diseases affecting iron metabolism.

MATERIALS AND METHODS

Subjects.—The *normal group* consisted of 8 young men between the ages of 20 and 40 years, 6 patients with psychoses, and 6 patients with other illness not affecting hematopoiesis. These patients were chosen on the basis of a normal history relative to iron metabolism, their state of good health, normal hematologic studies, and, when possible, the demonstration of adequate iron stores by marrow examination.

An *iron deficient group* consisted of 19 anemic patients who fulfilled the diagnostic criteria of having an absence of marrow hemosiderin and a depressed plasma iron level. Fourteen had microcytosis and 14 of 17 had hypochromia. Plasma iron was below 50 mg. per cent in all except one (subject 2) who had been placed on iron therapy 3 days preceding the determination. These patients were further divided into two groups, an uncomplicated and a complicated group. The uncomplicated was composed of those patients with no associated illness which might have altered iron metabolism and whose anemia responded promptly to oral iron. The complicated group consisted of those patients whose iron deficiency state, while fulfilling the criteria for iron deficiency, was refractory to oral iron therapy or those who had some disease which might be anticipated to alter absorption.

The *group with increased erythropoiesis* included 15 patients with various hematologic disorders, all having in common increased red cell marrow activity. Reticulocyte counts are included in this group to indicate the level of effective erythropoietic activity.

Decreased erythropoiesis was represented by 4 patients, all with marked reticulocytopenia and a significant degree of anemia; 3 of the 4 patients required transfusions.

Iron storage disease (idiopathic hemochromatosis) was represented by 5 patients who had been so diagnosed through histologic examination of liver biopsies.

Procedure.—Subjects were questioned concerning prior oral iron intake, diet, blood donations or transfusions, and associated disease. Blood studies were performed which included red cell count, hematocrit, hemoglobin, reticulocyte count, serum iron, and, when possible, marrow aspiration for hemosiderin.

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The iron absorption study was done at noon. Canned food materials were obtained for the test, and the same manufacturer's lot was used throughout the experiment. The test meal consisted of corned beef hash (200 Gm.), applesauce (200 Gm.), tomato juice (200 Gm.), soda crackers (13 Gm.), and cocoa (3 Gm.). The iron content of the meal was found by analysis to be 4.6 mg. A tracer dose of 50 μc Fe^{55} Cl^{+++} was administered halfway through the meal.

The absorption of radioiron from this meal was measured by the double isotope method of Saylor and Finch[2] in all studies except for those on 2 patients (61 and 50) who had negligible erythropoietic activity. The method consisted of administering one isotope of iron by mouth as ferric chloride and the other, bound to fresh plasma, intravenously. Two weeks after the test meal, samples of blood were obtained and a quantity sufficient to give adequate counts was electroplated on copper planchettes. The per cent of absorption was determined by comparing the relative amounts of the two isotopes in the blood to the doses administered.

A second method, designed to determine comparative absorption of iron on two occasions, was employed. Here, one isotope of iron was given with a test meal on one day, and a second isotope was administered with a test meal on the next day. This permitted comparison between a control absorption study and the situation to be evaluated. Further details of these methods and the stool recovery technique of measuring absorption are provided in the preceding article.[1]

Plasma iron was estimated by the method of Bothwell and Mallett,[3] or Kitzes, Elvehjem, and Schuette.[4] Bone marrow iron was estimated from sternal aspirate by the method of Rath and Finch.[5] Hemoglobin was determined as oxyhemoglobin in an Evelyn colorimeter and hematocrits were spun at 1,800 g for 30 minutes. Reticulocytes were stained with cresyl blue and the number per 1,000 erthrocytes enumerated.

TABLE I. ABSORPTION OF IRON GIVEN WITH A TEST MEAL IN NORMAL SUBJECTS

PATIENT NO.	SEX	PCV (PER CENT)	MCV CUμ	MCHC (PER CENT)	RETICULOCYTES (%)	PLASMA IRON (μG%)	MARROW FE (GRADE)	IRON ABSORPTION (PER CENT)
A. Normal Students								
35	M	48	89	33	1.5	115	–	16.6
34	M	47	89	33	1.7	154	1	2.6
31	M	46	87	36	1.4	137	1	12.8
32	M	45	84	34	1.4	102	–	3.6
109	M	47	86	33	1.0	125	–	2.9
110	M	46	91	30	1.2	109	–	4.1
111	M	46	98	31	1.8	135	–	1.7
112	M	47	98	32	0.2	91	–	6.5
B. Patients Convalescent From Nonhematological Disorders								
1	M	47	90	33	0.8	90	1	10.5
15	F	37	86	31	0.7	114	3	0.6
16	F	46	94	33	2.1	90	2	2.0
17	F	42	80	33	0.7	114	1	0.5
18	F	43	74	31	2.0	70	3	3.8
9	M	45	84	32	0.4	90	2	1.5
*C. Subjects With Psychoses**								
21	M	46	85	34	1.1	95	–	3.3
12	M	46	88	35	–	62	–	7.1
22	M	47	85	34	0.9	160	–	6.2
14	M	48	96	30	0.7	105	–	6.1
20	M	48	83	33	1.1	101	–	6.9
13	M	48	99	33	2.2	136	–	7.0
D. Patients With Pernicious Anemia in Remission								
54	M	40	88	38	1.2	123	3	5.5
57	F	43	80	33	1.1	74	–	0.6
56	F	38	78	35	1.3	82	–	6.5
55	F	38	89	37	1.1	59	–	8.6

*In contrast to other groups when radioiron was given with meals, in Group C radioiron was administered immediately preceding the regular hospital meal.

RESULTS

The absorption of radioiron given with a standard meal to 20 patients considered normal and 4 patients with pernicious anemia in remission is summarized in Table I. The average absorption was 5.3 per cent (S.D. ± 4 per cent). The maximal absorption was 16.6 per cent and the minimal absorption 0.5 per cent. There was no indication that variations from the mean in this group of 20 patients were related to age, sex, hematocrit, reticulocyte count, plasma iron, or marrow iron. No decrease of absorption was found in 4 patients with gastric achlorhydria (Group D).

To indicate to what extent composition of the diet might alter iron absorption from this test meal, supplements of ascorbic acid in amounts of 200 to 1,000 mg. were given to 4 subjects. While absorption was not appreciably increased with the 200 mg. dose, with amounts of 500 to 1,000 mg., absorption was doubled when compared to the control value of the same subjects (Table II).

TABLE II

SUBJECT	ASCORBIC ACID (MG.)	ABSORPTION (% OF ADMIN. IRON)	
		CONTROL	WITH ASCORBIC
Lo.	200	3.2	4.1
Ho.	500	4.2	9.0
Ha.	750	1.9	4.3
Bo.	1000	10.6	22.1

Iron Deficiency.—Absorption of radioiron from the test meal was studied in 7 female patients and in 1 male patient who had uncomplicated iron deficiency anemia (Table III, *A*). Absorption was increased above the average normal of 5.3 per cent in all these patients. The average absorption in the uncomplicated iron deficient group was 21.6 per cent.

A second group of 11 subjects was of interest in that absorption was depressed despite iron deficiency anemia (Table III, *B* and Fig. 1). Complicating conditions included malabsorption syndrome (nontropical sprue), myxedema, infection, gastrointestinal neoplasm, and previous gastrointestinal surgery. In 3 patients there was no response to oral iron but response did follow intravenous saccharated iron. In two patients who had had partial gastrectomy there was a response to a therapeutic dosage of oral iron salts. One patient (75) with no detectable associated disorder and with moderately severe iron deficiency anemia did not respond to oral iron, but had a normal response to intravenous therapy.

Increased Erythropoiesis.—Fifteen patients with increased marrow erythroid activity were studied (Table IV). Eleven of these had not been given oral or parenteral iron or transfused blood. They were considered to have normal body iron stores although it was appreciated that a shift in iron had occurred from red cells to tissues. The first 7 patients had pernicious anemia in relapse. More detailed hematopoietic studies, reported elsewhere on similar patients who also had B_{12} deficiency, indicated that despite low circulating reticulocyte counts total marrow erythropoiesis was approximately three times normal.[6] Absorption in the group of 7 patients averaged 5.7 per cent. The additional 4 patients with hereditary hemolytic anemia averaged 9.7 per cent

TABLE III. IRON DEFICIENCY

PATIENT NO.	DIAGNOSIS	SEX	PCV	MCV	MCHC	RETICULOCYTES	PLASMA P.I.	MARROW	ABSORPTION	RESPONSE TO ORAL FE THERAPY	RESPONSE TO ORAL FE I.V.
A. Uncomplicated											
58	Intestinal bleeding	F	38	80	25	0.9	27	0	38.7	+	
47	Hypermenorrhea	F	28	71	-	2.8	35	0	9.9	+	
1	Hypermenorrhea	F	27	78	29	2.7	20	0	22.0	+	
80	Bleeding peptic ulcer	M	26	114	33	1.2	25	0	22.0	+	
2	Multiple pregnancies on iron therapy	F	29	74	28	6.9	69	0	21.0	+	
76	Hypermenorrhea	F	35	55	25	-	7	0	17.6	+	
90	Bleeding ulcer	F	26	60		1.3	27	0	23.6	+	
70	Hypermenorrhea	F	29	89	25	5.6	10	1	17.7	+	
B. Complicated											
5	Idiopathic malabsorption	F	29	67	27	22.0	17	0	0.6	0	+
	Idiopathic malabsorption	F	25	71	28	2.1	29	0-1	0.5	0	+
49	Cancer of colon	F	27	70	30	2.0	20	0	2.7		
25	Low grade infection	F	24	82	30	1.2	36	0	2.6	0	+
39	Myxedema	F	30	65	33	1.6	20	0	2.7		(after thyroxin)
7	Postgastrectomy	F	33	61	28	-	32	-	0.6		+
68	Postgastrectomy	M	28	60	30	1.2	13	0	6.5 (suboptimal)		+
59	Postgastrectomy	M	28	71	28	0.8	18	0	5.0		
74	Postgastrectomy	M	21	54	35	2.2	12	0	1.3 (transfused)		
73	Postgastrectomy and gastrointestinal bleeding	F	28	80	30	0.6	24	0	2.3 (suboptimal)	+	+
75	Malabsorption of iron and uterine myomata	F	35	66	19	1.3	22	0	2.3	0	+
131	Myxedema	F	28	69		1.8			1.4		

TABLE IV. INCREASED ERYTHROPOIESIS

PATIENT NO.	DIAGNOSIS	SEX	PCV (%)	MCV	MCHC	RETICULOCYTES	P.I. MCG %	MARROW GRADE	ABSORP. (%)
A. Without Associated Iron Loading									
96	Pernicious anemia in relapse	F	36	118	--	0.6	80	-	0.3
102	Pernicious anemia in relapse	F	29	112	34	0.5	124	2+	3.4
108	Pernicious anemia in relapse	M	17	102	30	0.4	141		6.8
106	Pernicious anemia in relapse	F	34	-	--	0.8	42	2+	0.8
94	Pernicious anemia in relapse	M	26	126	30	0.7	197	2+	22.5
93	Pernicious anemia in relapse	F	15	134	37	-	162	3+	5.7
120	Pernicious anemia in relapse	M	26.5	101	36	0.3	137		0.6
121	Hereditary nonspherocytic anemia	M	41.4	-	--	7.2	60	3+	8.4
117	Hereditary nonspherocytic anemia	M	--	-	--	1.9	159		5.6
114	Hereditary spherocytic anemia	M	37	86	--	2.1	147		3.5
101	Hereditary nonspherocytic anemia	M	37	85	34	12.0	129		21.3
B. With Associated Iron Loading									
98	Sickle cell anemia (last transfusion 2 months prior)	F	29	82	33	5.6	166	5+	13.5
122	Paroxysmal nocturnal hemoglobinuria	M	36.5	96.7	31.8	13.6	191	5+	2.5
115*	Thalassemia minor	F	37	76	28	1.9	84	4+	1.0
51*	Thalassemia minor	F	29	71	31	2.6	70	5+	1.1

*Survival time by Cr^{51} technique was ½ time of 13½ days and 14½ days.

TABLE V. DECREASED ERYTHROPOIESIS

PATIENT NO.	DIAGNOSIS	SEX	PCV (%)	MCV	MCHC	RETICULOCYTES	PLASMA IRON (μg %)	MARROW FE GRADE	ABSORP. (%)
A. Without Associated Iron Loading									
61	Aplastic anemia	M	15	81	33	0.1	87		1.4*
B. With Associated Iron Loading									
46	Uremia 44 Tx	M	19	101	36	0.1	85	4	0.13
116	Refractory anemia 7 Tx	F	30	90	30	0.3	46	4	0.59
50	Refractory anemia 200 Tx	M	22	81	39	0.2	260	–	0.0*

*Determination done by stool method. (These patients had no demonstrable activity in circulating blood.)

absorption. Four patients had been repeatedly transfused and had heavy hemosiderin deposits in the marrow. Absorption studies in these patients showed a low absorption in 3 and a somewhat elevated absorption in the fourth, with a group average of 4.5 per cent. As shown in Fig. 2, the absorption in this group of 15 patients with hemolytic anemia was only slightly higher than the normal patients studied.

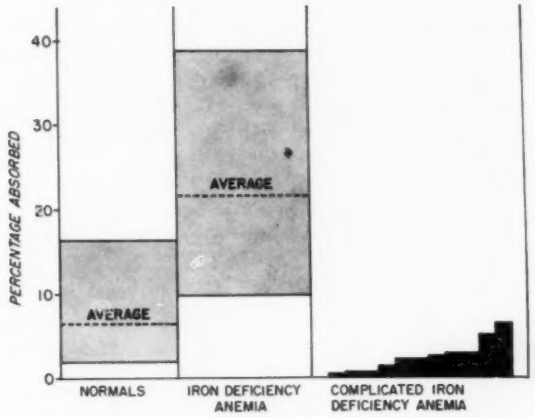

Fig. 1.—Absorption in complicated patients with iron deficiency anemia is shown by the solid areas on the right, as compared with absorption observed in normal subjects and patients with uncomplicated iron deficiency anemia.

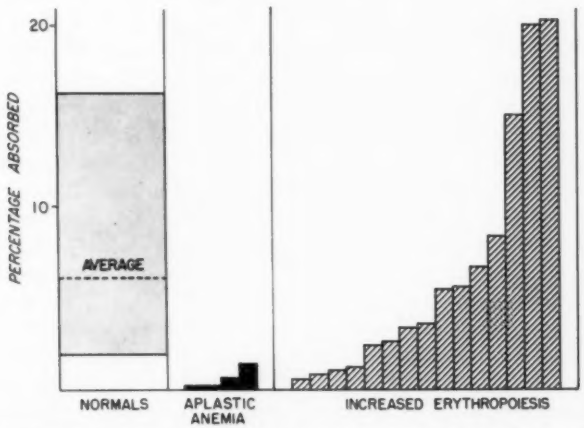

Fig. 2.—Relation of iron absorption to erythropoiesis. The normal range of absorption is shown on the left. Solid black areas indicate the per cent of absorption in 4 patients with aplastic anemia. Absorption of iron by 15 patients with increased erythropoiesis is indicated by the cross-hatched columns on the right.

Decreased Erythropoiesis.—Four patients with decreased red cell production were studied (Table V). One of these patients had had no previous transfusions. The others had been transfused frequently. Absorption, measured by

the double isotope method in all and the stool method as well in the 2 patients who had a negligible red cell function (61 and 50), indicated marked depression in iron absorption in all 4 patients.

Idiopathic Hemochromatosis.—Absorption studies (Table VI) were carried out before treatment in 5 patients with iron storage disease. Their absorption varied from 4.7 to 17 per cent (average of 10.6 per cent). While this is not greatly above the normal it becomes particularly significant when the large iron stores of these patients are considered. Furthermore, absorption was increased to 29.7 per cent following multiple phlebotomies at a time when the serum iron was still elevated or normal and tissue iron stores were still greatly increased. This increase was greater than that seen in cases of noncomplicated iron deficiency anemia or in the group of phlebotomized normal subjects mentioned in the previous article.

TABLE VI. HEMOCHROMATOSIS

PATIENT	DIAGNOSIS	SEX	PCV (%)	MCV	MCHC	RETICULOCYTES	P.I. MCG %	MARROW FE GRADE	ABSORP.
A. Before Phlebotomy Treatment									
48		M	42	90	33	1.0	318	–	4.8
	Met.	M	42	102	33	1.9	245	4	12.9
81		M	47	92	33	1.1	260	4	17.0
124		M	46	–	–	1.2	212	–	11.4
38		M	76	90	36	0.7	285	4	7.0
B. After Repeated Bleeding, but Not Iron Depleted									
38A		M	43	87	34	2.6	105		29.7
48A		M	38	89	30	2.0	262		29.7
88		M	42	87	34	2.0	192		29.4

DISCUSSION

It is generally accepted that the amount of iron absorbed from food is dependent on both the individual's capacity to absorb iron and the "availability" of the dietary iron ingested. The absorption of food iron may differ greatly according to the type of food in which it is found. This is well documented by the studies of Chodos and associates,[7] Walsh and associates,[8] and Moore and associates[9] in which radioiron was incorporated into several food articles in its natural form and its absorption measured. However, these reports indicate the absorption of iron from only a few foods and no attempt has been made to label an entire diet. Beyond this, the blocking effect of such substances as phosphates[10] and phytates[11] and the enhancement of absorption by reducing substances such as ascorbic acid must be considered.[12]

It becomes apparent, if one assumes that isotope methods are essential for precise measurement of absorption, that not only is the isotope labeling of an entire diet a formidable task, but that an arbitrary diet must be selected regardless of the extent of labeling. After considering these problems it was decided to employ a standard meal to which Fe^{55} Cl was added in tracer amount. It was assumed that this iron would mix with a considerable portion of food iron which would be converted to chloride salt within the stomach. Thus, while not representing all of the dietary iron, this would represent a large portion of ingested food iron.

The average absorption in normal subjects given this tagged test meal (Table I) was 5.3 per cent, which might be extrapolated to a daily absorption of 0.8 mg. from a diet containing a total of 16 mg. of iron. The results are in general similar to other studies employing food and radioiron techniques. Sharpe and associates[11] found an average absorption of 4.4 per cent in adolescent boys fed a meal of milk, rolled oats, tomato juice, white bread, omelet, and radioiron. Chodos and associates[7] found 0.5 to 5 per cent absorption of radioactive iron from eggs, 0.8 to 1.6 per cent from Swiss chard, and 0.8 to 0.9 per cent from beet greens. Moore and Dubach[9] found 1 to 8 per cent from tagged eggs and an average of 6 per cent from liver. Walsh and associates[8] found 3 per cent from hemoglobin iron and 8 per cent from liver and kidneys. These figures are also in general agreement with the excretion studies of Dubach and co-workers[13] and the careful balance studies of Johnston and associates[14] which estimate an iron turnover in man of approximately 0.5 to 1.5 mg. per day.

A comparison of the present results with those obtained previously when iron alone was given[1] indicates that the presence of food reduces absorption by approximately one half. This effect has been pointed out by others.[7, 10, 11] Studies with ascorbic acid reported here (Table II) and presented by others[12] suggest that the action of ascorbic acid is concerned at least in part with this blocking mechanism of food since ascorbic acid is less effective with iron salts alone.[1]

Results in 4 subjects with histamine-fast achlorhydria show no significant deviation from the normal, lending further support to the contention of Moore and Dubach[9] that gastric acidity plays no demonstrable role in the absorption of iron from food.

Variations from 0.5 to 16.6 per cent in absorption from this test meal observed between individuals in the normal group are unexplained, since no correlation was observed between these results and the hematologic measurements, plasma iron level, or marrow iron. Such differences, however, do point out the difficulty of evaluating factors affecting iron absorption in the normal subject.

Erythropoietic activity has been shown to affect absorption of iron salts.[1] With decreased red cell production, iron absorption was depressed, whereas increased erythropoiesis was associated with enhanced iron uptake. Consistent with these results the absorption of iron from food was depressed in 4 subjects with aregenerative anemia to between 0 and 1.4 per cent and had an associated increase in iron stores. Patients with increased erythropoiesis showed less consistent results (Fig. 2). Discounting the subjects who had increased body iron stores due to transfusion, the remainder showed an absorption of 7.2 per cent, only slightly above the normal average. This group was heavily weighted with patients having pernicious anemia in relapse. Their erythroid marrow is know to be hyperactive and their plasma iron turnover three times normal,[6] yet iron absorption was not significantly increased (Table IV, *A*).

Patients with other types of hemolytic anemia had an average absorption somewhat above the normal range (9.7 per cent as compared to the normal average of 5.3 per cent). While in better controlled animal studies it has

been possible to demonstrate in a variety of hemolytic anemias a significant increase in absorption, results in man are less convincing. It is possible that the present clinical data are inadequate, since relatively few patients with maximal erythropoiesis were studied; yet there was no obvious correlation between degree of erythropoiesis and the level of absorption (see Patients 122, 101, 94, and 106). It is also likely that increased iron stores, which have been shown to be able to counteract the effect of increased erythropoiesis on iron absorption, might be of importance in keeping absorption within normal range. In an attempt to evaluate this point, a comparison was made between patients where iron stores were not known to be enlarged through therapy (Group A of Table IV), and patients having had previous transfusions or oral iron (Group B). While absorption in Group B was 4.5 per cent as compared to 7.2 per cent in Group A, individual variations were so great as to make this of little significance. Further attempts by history and marrow examination for hemosiderin were not helpful in bringing individual variations into line. These studies, however, would suggest that iron overload from diet is unlikely in hemolytic anemia and that it should not occur in anemias with hypoplastic marrow. It follows that those cases reported to show such overload may be presumed to be the result of long continued oral iron therapy or blood transfusions.

The second internal factor of some consequence in the regulation of iron absorption is the state of iron stores.[1] In the presence of iron deficiency anemia, where there was considered to be both depleted iron stores and increased marrow capacity to assimilate iron, absorption of radioiron from the test meal was increased. The increased absorption of iron in iron deficiency has been consistently demonstrated both with iron salts[15, 16] and with food iron.[7, 12] In our own studies[1] absorption of 4 to 5 mg. of iron salts in the normal subject was estimated at about 15 per cent as compared to 80 per cent in the iron deficient subject. At a similar iron dose, absorption from food was 5.3 per cent in the normal and approximately 22 per cent in the iron deficient patient. It is also of interest to estimate daily food iron absorption from the later data. Assuming a total dietary iron intake of 15 mg. per day and an absorption of 22 per cent, daily absorption would be 3.3 mg. This is quite similar to the figure of 3 to 4 mg. per day calculated previously from the iron balance of phlebotomized patients with polycythemia vera.[17]

While experimental evidence is conclusive in showing a depression of absorption as a result of iron overload, no uncomplicated patients were available on whom such observations could be made. However, the data on patients with combined hemolytic anemia and iron overload or decreased erythropoiesis and iron overload tend to substantiate this. Information now available does not permit a conclusion as to whether absorption is altered by small increases or decreases in iron stores.

While an extreme degree of iron overload exists in idiopathic hemochromatosis, this must be regarded as a special condition. Here it has been recognized that excessive absorption is responsible for the iron surplus, and this excessive absorption has been demonstrated.[7, 8, 18] Observations on the 5 subjects studied here indicate that despite massive iron stores and elevated plasma iron, the

average absorption was over 10 per cent. Following phlebotomy with resultant increase in erythropoiesis, the average absorption was increased to 29 per cent in spite of the fact that iron stores were still excessive and that plasma iron level was normal or elevated. It should be noted that this figure exceeds that found in uncomplicated iron deficiency anemia or in phlebotomized subjects. These studies, in keeping with observations of Chodos and associates[7] and Peterson and co-workers,[19] indicate that the state of iron stores and the level of erythropoietic activity may influence absorption in the patient with hemochromatosis as well as in the normal subject. The basic level of absorption in idiopathic hemochromatosis, however, is considerably above that found in normal man and accounts for the gradual accumulation of excessive iron in the body.

Factors other than decreased erythropoiesis or increased iron stores may decrease iron absorption. Such decreases are more clearly demonstrated in the phlebotomized, or better yet, in the iron deficient subject. In this study there were 11 patients who did not show the expected increase characteristic of iron deficiency. This was a selected group and does not in any way reflect the incidence of malabsorption of iron. Etiologic factors included (1) idiopathic steatorrhea, which has been previously demonstrated to interfere with iron absorption,[20] and to be responsible for perhaps one half of the instances of refractiveness to oral iron therapy; (2) infection, which has been shown to block not only mucosal transfer of iron,[21, 22] but the transportation of iron across the placenta and reticuloendothelial cells[23]; (3) myxedema, where the defect is reversible with therapy; and (4) the partial gastrectomy state, where a curious discrepancy exists between response to oral iron salts[24] and lack of absorption of food iron.[7] In view of the importance of time of exposure to the mucosa,[25] it might be suggested that the rapid transit of iron in a bolus of food might produce this abnormality.

Finally, a study of the absorption of iron in the various patients studied showed no correlation with the hemoglobin level of the patient. Thus it may be concluded that anemia per se plays no role in the regulation of iron absorption.

SUMMARY

The absorption of iron in food was studied by the administration of a test meal to which a tracer dose of $FeCl_3$ was added. A double isotope technique was employed to quantitate absorption.

The absorption of iron from food by both normal and iron deficient subjects was lower than that seen when iron salts alone were given. Supplemental ascorbic acid diminished this difference.

In keeping with previous studies employing iron salts, it was found that absorption from diet also varied inversely with iron stores and directly with the degree of erythropoiesis. However, increases in hemolytic anemia were usually not significant, and it seems unlikely that iron overload from dietary sources will occur in either aplastic or hemolytic anemia.

Studies in idiopathic hemochromatosis before and after phlebotomies indicate excessive absorption in these patients under all circumstances.

In addition to excessive iron stores and decreased erythropoiesis there are conditions which may act at the mucosal level to impair iron absorption. Idiopathic steatorrhea, infection, myxedema, and the postgastrectomy state have all been shown to result in decreased absorption by the iron deficient patient.

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LOWERED GLUCOSE UTILIZATION, PHOSPHATE UPTAKE, AND REDUCED GLUTATHIONE CONTENT OF ERYTHROCYTES FOLLOWING BILATERAL NEPHRECTOMY

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With the Assistance of B. Brooks

FOLLOWING bilateral nephrectomy (dog and rabbit) an anemia which is partly hemolytic develops.[1-3] Observations on the lifespan of erythrocytes following renal ablation of the dog[3] indicate the development within 4 days of a shortened erythrocytic lifespan. In this discussion we present data concerning the glucose utilization, phosphate uptake, and reduced glutathione (GSH) content of erythrocytes 4 days following bilateral nephrectomy of the dog.

METHODS

Thirty normal mongrel dogs (average weight 9.3 kilograms) of either sex were used. Both kidneys were removed at the same time by the anterior abdominal approach while the animal was under ether anesthesia. Following the nephrectomies 10 animals received a diet containing no protein, 10 animals received no diet, and 10 animals received a protein or amino acid containing diet. The caloric intake varied between none to 25 calories per kilogram per day (47 per cent protein, 27 per cent fat, and 26 per cent carbohydrate plus added vitamins as described[4]). In addition, all animals were given 16 ml. of physiologic saline per kilogram per day by the intravenous route. The saline was determined by experience to improve the well-being of the animal and seemed to prevent vomiting.

Duplicate analyses were performed on each sample. These duplicate values showed minimal variation and were averaged for the final values. Control samples were obtained prior to the nephrectomies and the test samples were obtained 4 days after the renal ablation. Ten milliliters of blood was drawn from the femoral artery directly into a syringe containing a drop of heparin solution. The sample was immediately chilled in ice and the analyses were begun within a short time.

The glucose utilization was determined according to the approach described by Hollingsworth,[5] which utilizes the Somogyi method for glucose.[6] The inorganic phosphate uptake was measured by the use of P^{32} containing orthophosphate as described by Feinstein, Gordon, and Bethard.[7] On 5 occasions, presented separately, the phosphate uptake was measured according to the method described by Prankerd.[8] The reduced glutathione content was determined according to the technique of Grunert and Phillips.[9]

RESULTS

The data in Table I are paired data referring to the control values before nephrectomy and the value 4 days after nephrectomy. A "t" test was used to

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test whether or not the mean difference between the control and 4-day values differed significantly from zero. The statistical summary is given in Table II.

TABLE I. PAIRED DATA (CONTROL AND 4 DAYS FOLLOWING BILATERAL NEPHRECTOMY) FROM THE 85 EXPERIMENTS. EACH MEASUREMENT REPRESENTS THE AVERAGE OF CLOSELY AGREEING DUPLICATE ANALYSES. THE DATA ARE ANALYZED STATISTICALLY IN TABLE II

DOG NO.	WT. (KG.)	GLUCOSE UTILIZATION (MG./100 ML. R.B.C.)		P^{32} UPTAKE (PER CENT)		GSH CONTENT (MG./100 ML. R.B.C.)	
		CONTROL	4 DAYS	CONTROL	4 DAYS	CONTROL	4 DAYS
567	12.0	48.7	45.6	13.6	11.7	38.8	34.4
568	8.6	48.4	52.6	12.9	11.1	38.4	31.6
573	8.3	47.1	34.9	9.2	7.8	38.5	26.1
574	10.5	52.5	59.6	11.4	10.5	37.3	26.1
575	8.3	44.3	59.3	16.1	11.1	43.3	36.3
576	10.0	53.9	60.3	12.1	11.4	42.4	36.8
577	9.7	38.3	35.1	13.6	10.3	54.8	39.1
578	11.8	46.4	37.3	14.5	12.2	40.5	32.1
588	10.0	54.6	34.0	13.2	6.5	32.2	29.5
589	13.5	53.9	42.9	12.2	6.4	38.2	22.8
596	9.5	48.7	34.7	10.0	7.5	47.6	39.8
597	8.2	41.8	30.2	9.7	6.1	56.4	29.2
598	6.8	46.2	31.9	11.1	9.7	41.0	27.4
602	10.5	51.1	35.5	11.0	8.7	48.2	30.5
631	12.0	62.4	30.0			48.9	30.9
579	5.8	73.9	68.9	16.8	8.2	39.1	20.7
580	8.4	51.3	39.6	15.8	12.7	35.6	28.5
581	10.0	58.1	46.5	14.8	12.1	54.6	40.7
583	8.2	53.0	42.4	16.2	8.1	33.9	28.4
584	8.7	55.8	42.8	14.1	6.5	38.4	23.6
605	7.3	50.0	36.5	12.4	9.5	38.9	12.6
606	6.1	55.0	34.0	11.0	8.4	38.4	22.1
610	5.7	54.5	39.4	11.5	9.8	55.8	40.9
565	11.0	60.7	61.6	16.2	12.0	48.8	42.8
566	12.5	50.1	49.7	14.8	13.5	57.2	38.4

TABLE II. STATISTICAL SUMMARY OF THE VALUES OF TABLE I

VALUES	GLUCOSE UTILIZATION	P^{32} UPTAKE	REDUCED GLUTATHIONE CONTENT
N	25	24	25
$\bar{x}$	8.24	3.43	12.24
$\sigma\bar{b}$	2.006	0.477	1.03
t	4.11	7.19	11.88
P	<0.001	<0.001	<0.001

The results for all 3 measurements are significantly different from zero, thus indicating a significant decline in all three biochemical measurements.

The lowering of the phosphate uptake by the erythrocytes is supported further by the data from the 5 animals for which the Prankerd method[8] was used. Table III contains these data as well as additional data on the glucose utilization and GSH content.

In the experiments of Table III repeated control measurements and measurements at 8, 16, 24, 72, and 96 hours following the nephrectomies were obtained. The data suggest beginning of lowering of the values by 24 hours and a distinct lowering by 72 to 96 hours. The lowered phosphate uptake is more definitely demonstrated by the Prankerd method which uses larger volumes of erythrocytes agitated and oxygenated in the Warburg apparatus.

TABLE III. IN THESE EXPERIMENTS THE GLUCOSE UTILIZATION, PHOSPHATE UPTAKE, AND GSH CONTENT WERE DETERMINED WITHIN 8 AND 96 HOURS FOLLOWING THE RENAL ABLATION. MULTIPLE BASE-LINE VALUES, EACH CHECKED BY DUPLICATE ANALYSIS, WERE SIMILAR. THE DATA SUGGEST CHANGES WITHIN 24 HOURS OF THE RENAL ABLATION

DOG NO.	WT. (KG.)	TIME (HOURS)	GLUCOSE UTILIZATION (MG./100 ML. R.B.C.)	P^{32} UPTAKE (PER CENT)	REDUCED GLUTATHIONE (MG./100 ML. R.B.C.)
614	9	0	53.0	34.3	42.7
		0	52.6	35.0	42.7
		0	51.6	37.9	44.0
		Av.	52.4	35.6	43.1
		8	50.0	31.9	43.7
		16	48.6	20.8	42.4
		24	47.1	20.4	36.7
616	11	0	57.9	26.8	41.1
		0	59.0	24.3	40.6
		Av.	58.5	25.6	40.9
		24	55.3	11.0	34.0
		72	42.9	9.7	20.8
626	11.5	0	65.9	34.8	53.6
		24	67.6	30.5	50.3
		72	56.6	20.4	22.1
630	10.5	0	59.3	23.9	55.0
		24	53.2	19.6	52.7
		72	48.0	16.8	37.6
631	12	0	61.0	32.3	46.7
		0	63.7	28.8	51.1
		Av.	62.4	30.6	48.9
		24	59.4	20.1	39.0
		96	30.0	19.9	30.9

DISCUSSION

Under conditions similar to those of the present experiments it has been demonstrated that the red blood cell volume is lowered, the serum bilirubin is elevated, the stool urobilinogen is increased, and the erythrocyte lifespan is shortened.[1, 3] At autopsy such animals have shown erythrophagocytosis by reticuloendothelial cells of lymph nodes, spleen, and liver.[1] Thus, the biochemical changes of the erythrocytes noted herein (lowered glucose utilization, lowered phosphate uptake, and lowered GSH content) occur in the setting of a hemolytic state. The changes indicate rapidly developing alterations of the erythrocytes attendant on the renal ablation.

From a theoretical view one would expect these biochemical changes to be related to the erythrocytic injury which determines the phagocytosis of red cells and thus their shortened lifespan. Since the shortened lifespan appears to be mediated mainly by erythrophagocytosis, a surface or membrane alteration of the erythrocyte is indicated.

SUMMARY

Following bilateral nephrectomy of the dog, under conditions associated with a hemolytic state, the glucose utilization, phosphate uptake, and reduced glutathione content of erythrocytes are significantly lowered. These biochemical changes occur within a relatively short time following the renal ablation

and may be taken as an indication of a rapidly developing erythrocytic alteration. These biochemical changes may be related to the injury of the cell which leads to erythrophagocytosis and a shortened lifespan of the erythrocytes in this state.

I wish to thank Dr. J. A. Stirman for assistance in the preparation of this material.

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ZONE ELECTROPHORETIC STUDIES ON THE DISTRIBUTION OF THE L.E. FACTOR IN THE SERUM PROTEINS AND URINARY PROTEINS FROM PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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THE L.E. cell phenomenon was originally[1] described in bone marrow preparations from patients with systemic lupus erythematosus (S.L.E.) The observation of Haserick and co-workers[2-6] that the L.E. cells could be induced in vitro by normal leukocytes in the presence of serum from the patient indicated that the phenomenon apparently was due to a pathological component in the serum of the patient. This so-called L.E. factor which was shown to be nondialyzable and independent of complement was destroyed over 56° C. but could be kept in the icebox for months.[6, 7]

As the S.L.E. patients usually have a considerably increased gamma globulin fraction in the serum it seemed plausible to assume a correlation between the L.E. factor and the gamma globulins. Haserick, Lewis, and Bortz,[6] after separating serum proteins from S.L.E. patients in free electrophoresis, were able to induce the L.E. cell phenomenon with the gamma globulin but not with any other fraction. After fractionation of S.L.E. plasma with ammonium sulfate Lee, Michael, and Vural[7] reported that the greatest number of L.E. cells was formed by the protein fractions with the highest gamma globulin content.

As the recent development of the zone electrophoresis technique allows a simple and more complete separation of the proteins than with the classical electrophoresis analysis it seemed worth while to reinvestigate the electrophoretic behavior of the L.E. factor in the S.L.E. serum. Being of great theoretical interest the urinary proteins from a S.L.E. patient were also analyzed, which as far as we know have not been subjected to investigations of this type before.

MATERIALS

The serum for the analysis was taken from a 53-year-old woman with a systemic lupus erythematosus of long duration mainly involving the kidneys and the vascular system. Death was due to massive internal haemorrhages. The autopsy confirmed the diagnosis of S.L.E. Repeated L.E. cell tests were strongly positive.

The urine for the analysis was obtained from a 32-year-old man with cutaneous manifestations of S.L.E. for 7 years. The last 2 years he gradually developed a nephrotic

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syndrome with massive proteinuria, hypoproteinemia, and edema. The condition was complicated by a pleural effusion and ended subsequently with death in signs of renal insufficiency. The L.E. cell phenomenon could easily be induced by finger blood as well as with pleural fluid and untreated urine.

Before the analysis 100 ml. of urine was dialyzed against running tap water for 24 hours and then against 15 per cent high molecular dextran for another 24 hours to a final volume of about 4 ml. and a protein concentration of about 3 Gm./100 ml.

METHODS

The Zone Electrophoresis.—The electrophoretic separation was carried out by zone electrophoresis in starch medium (Kunkel and Slater[8]) with the column technique elaborated by Carlson[9] (1954).

Barbital buffer of pH 8.6 and ionic strength 0.1 was used. One milliliter of the sample was applied to a column which had an internal diameter of 20 mm. and was equipped with an external cooling mantle. The separation was performed at +4° C. for 20 hours with a current of 25 mA. After the electrophoresis the column was eluted with buffer. Eluates of 1.4 ml. were collected, 0.1 ml. portions of which were analyzed as to the protein content according to the modified Folin reaction (Lowry and associates[10]). The remainder of the eluates were tested for the presence of the L.E. factor. The serum fractions were tested without previous concentration but the urinary proteins had to be dialyzed against running tap water for 24 hours and then against 15 per cent high molecular dextran for another 24 hours. The proteins were then dissolved in smallest possible volume of saline.

Estimation of the L.E. Factor.—The eluates after zone electrophoresis were tested for the presence of the L.E. factor according to a method described by Snapper and Nathan[11, 12] and modified by Möllerberg[13] (1956).

Three to four drops of finger blood from a normal person were put on a thoroughly cleansed slide. The preparation was then incubated in a moist chamber (Petri dish, moistened filter paper on the bottom, the slide on a pair of glass rods) for 60 min. at 37° C. The leukocytes will migrate through the coagulum on to the slide forming a layer under the blood drop. After the incubation the coagulum was carefully removed by gentle washing of the slide with a mixture of equal parts of saline and distilled water. Practically nothing but leukocytes were then left on the slide which was allowed to dry at room temperature. Three to four hours later a drop of the protein solution to be tested was put on the leukocyte layer of the slide, which was incubated in a moist chamber for 30 min. After discarding excess of the test solution, three to four drops of normal finger blood were put on the slide which once again was incubated in a moist chamber, this time for two hours. Living leukocytes will now migrate on to the slide and phagocyte the old leukocytes thus being able to form L.E. cells. The coagulum was removed as before. Fixation and dyeing of the preparation was made as for differential count of leukocytes. The whole slide which contained approximatively 3,000 leukocytes was examined under high magnification. The number of L.E. cells was counted. No attention was paid to so-called Tart cells as these may be seen in normal preparations. The L.E. cells were not given in exact numbers which might give the estimation a false impression of accuracy but were roughly estimated as "occasional L.E. cells," "several L.E. cells," and "abundant L.E. cells."

RESULTS

The electrophoretic patterns of the serum proteins and the urinary proteins are given in Figs. 1 and 2, respectively. The fast-moving component is to the left in the figures. The serum proteins show a decrease in the albumin fraction and an increased amount of heterogenous gamma globulins. The L.E. factor is found in the fractions corresponding to gamma globulins of slow

mobility. Essentially the same result is to be seen on the diagram of the urinary proteins although the L.E. factor here shows a slightly wider distribution among the gamma globulins.

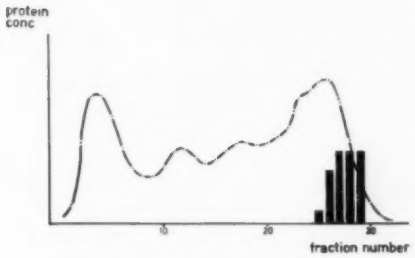

Fig. 1.

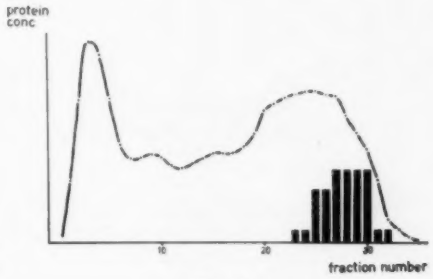

Fig. 2.

Fig. 1.—The localization of the L.E. factor in the zone electrophoretic pattern of the serum proteins from a case of systemic lupus erythematosus. The albumin is to the left in the figure. The L.E. factor is visualized by black bars the heights of which are approximatively proportional to the number of L.E. cells found in testing the corresponding fraction.

Fig. 2.—The localization of the L.E. factor in the zone electrophoretic pattern of the urinary proteins from a case of systemic lupus erythematosus. For explanation, see Fig. 1.

DISCUSSION

The protein component responsible for the L.E. cell phenomenon has thus been localized to the gamma globulin fraction of the S.L.E. serum which confirms the earlier observations made by, among others, Haserick. The additional information gained in this investigation is, however, that the L.E. factor appears to have a slower mobility than the mean mobility of the gamma globulins. This indicates that the ability to induce the L.E. cell phenomenon is not a common property of the gamma globulins in S.L.E. but is apparently confined to a specific protein component. A theoretical objection to this conclusion is that the L.E. factor might be evenly distributed among the gamma globulins but that the beta globulins overlapping the fast-moving part of the gamma globulins contain a specific inhibitor to the L.E. factor. It has, however, been found that the L.E. cell phenomenon is not inhibited in the presence of the beta globulins.

As antibodies of different kinds belong to the gamma globulins it has been suggested that the L.E. factor should be an antibody. This theory is supported by experimental work[14, 15] but has also met with considerable criticism.[16] In this connection it might be of interest to mention a few investigations of the electrophoretic behaviour of some antibodies. After continuous zone electrophoresis of acute phase protein, Hedlund and Brattsten[17] found that this antibody showed a fairly even distribution among the gamma globulins. The antibody to polio virus type I has recently[18] been localized to the slow-moving gamma globulin fraction.

The finding of the L.E. factor in the urinary proteins is in accordance with earlier observations.[19, 20] It is, however, notable that the L.E. factor even after passage through the kidneys has essentially the same electrophoretic distribution as in serum.

SUMMARY

After zone electrophoretic separation of the serum proteins and urinary proteins from patients with systemic lupus erythematosus the L.E. factor was found to migrate as a gamma globulin of lower mobility than the mean mobility of the gamma globulins.

ADDENDUM

Since this paper was prepared Ziff and collaborators in a personal communication have reported similar results as to the zone electrophoretic behaviour of the L.E. factor.

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THE EFFECT OF 5-HYDROXYTRYPTAMINE (SEROTONIN) ON THE RESPIRATION OF NONASTHMATIC AND ASTHMATIC SUBJECTS. I.

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SOME descriptions of the clinical manifestations of the syndrome of malignant carcinoid have included wheezing respiration as a common finding, and this has been ascribed to the liberation of 5-hydroxytryptamine by the tumor.[1-3] In view of a possible bearing on the genesis of bronchial asthma and obstructive pulmonary disease, the following studies were undertaken to assess what effect, if any, administered 5-hydroxytrptamine might have on the respiration of normal and asthmatic subjects.

METHODS

Twenty-two subjects without evident pulmonary disease, ranging in age from 22 to 55 years, and 8 asthmatic patients ranging in age from 22 to 45 years, were studied. The asthmatic patients all had had severe bronchial asthma for 5 or more years, were skin sensitive to various allergens and were all being treated by injections of allergenic extracts at the time of these studies.

The patients were placed in the horizontal position, lying comfortably. They were connected by means of a soft rubber mouth piece to a closed spirometric system with oxygen flowing into it so as to replace that taken up by the subject. Recordings were made simultaneously on two kymographs running at 60 cm. per minute and at approximately 5 cm. per minute. Carbon dioxide was removed by means of a soda lime scrubber. Continuous recordings of respiration were made before, during, and after injection of 5-hydroxytrytamine (5-HT) in a dosage range from 0.5 to 1.5 mg., given directly and rapidly into an anticubital vein.

Thirty such injections were made in 22 nonasthmatic subjects and 15 in 8 asthmatic patients.

RESULTS

Nonasthmatic Subjects.—A response was elicited in all subjects, the nature of which, however, could not be predicted. In 19 of the 22, rapid infusion of the drug resulted in an increase in the rate and depth of respiration lasting from 20 to 40 seconds. A typical response is shown in Fig. 1, *A*. The respiratory effects were associated with numbness and tingling of the extremities, flushing of the face, a burning sensation in the throat, and an occasional crampy abdominal pain.

In the remaining 3 nonasthmatic subjects, the response noted was that of sudden apnea followed by a short period of hyperpnea. Systemic sensations were no different from that noted in those with the hyperpnea alone. A typical response is shown in Fig. 1, *B*.

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Asthmatic Patients.—In 8 asthmatic patients, the respiratory effects and systemic sensations were no different than those in the nonasthmatic group except that all exhibited hyperpnea and tachypnea and apnea did not occur.

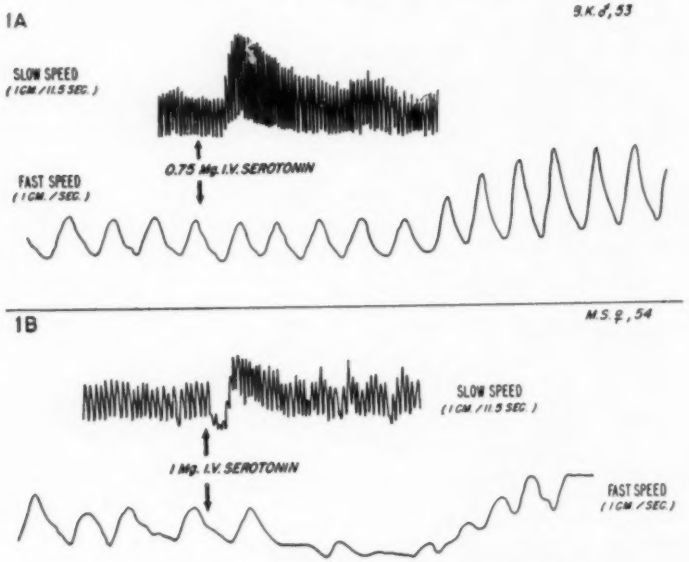

Fig. 1.—Respiratory response to intravenous serotonin.

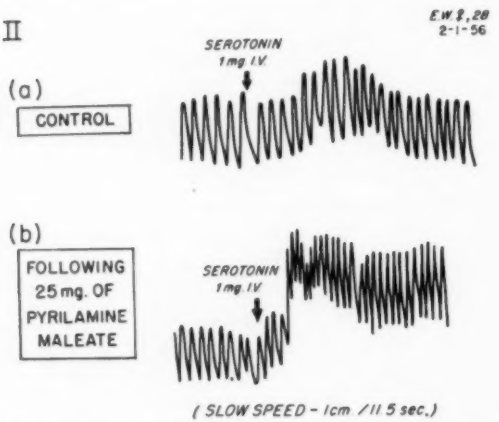

Fig. 2.—Effect of pyrilamine maleate on the respiratory response to serotonin.

There was no evident wheezing or any subjective or objective evidence of increased bronchial obstruction, even with very marked systemic effects at the higher dosage range. A typical response is shown in Fig. 2, *A*.

Observations on the Mechanism of the Respiratory Effects of 5-Hydroxytryptamine.—

1. *Histamine and antihistaminic drugs:* Page and McCubbin concluded that in dogs and cats, injected 5-HT caused histamine release which mediated the initial fall in blood pressure.[4] Feldberg and Smith independently described histamine release by 5-HT in perfusion experiments.[5] In view of this and in an effort to discover any similarity of respiratory action between 5-HT and histamine, 0.05 mg. histamine base was given by intravenous injection to several nonasthmatic subjects. No change in respiration was noted, but there was headache, sweating, flushing of the face, and a fall in blood pressure. Each subject easily distinguished these systemic effects from those caused by 5-HT. As previously noted by others,[6] intravenous histamine in asthmatic subjects causes severe bronchial obstruction and as this has been confirmed many times, it was not repeated in our study.

Antihistaminic drugs were given intravenously in both the nonasthmatic and asthmatic groups and the effect of a subsequent injection of 5-HT observed. If any of the respiratory effects noted were due to histamine release, previous intravenous injection of an antihistaminic drug would be expected to diminish the response. A dose of 5-HT capable of producing a respiratory and systemic effect was given and the response noted. This was then followed by an intravenous dose of pyrilamine maleate (25 mg.). This drug was chosen because of all the drugs of this group, it is the one which possesses the least parasympatholytic activity.[7]

In no instance did the injection of pyrilamine maleate decrease the respiratory effects of 5-HT. On the other hand, in 11 of 12 instances, potentiation occurred as illustrated in Fig. 2, *B*. The antihistaminic drug itself produced no effect other than mild sedation and some decrease in both the rate and depth of respiration.

The potentiation of the respiratory effect of 5-HT by intravenous pyrilamine maleate may be analogous to the potentiation of the pressor effect of epinephrine by antihistaminic drugs described by Loew.[8] Both 5-HT and epinephrine are degraded by monamine oxidase, an enzyme present in high concentration in the lung.[9] Monamine oxidase has been shown to be inhibited by many antihistaminic agents[10] and since pyrilamine maleate potentiated the respiratory effect of intravenously injected 5-HT, the effect of this antihistaminic drug on monamine oxidase and a resulting suppression of the destruction of 5-HT would explain the results obtained. But since histamine is not degraded by monamine oxidase, our studies therefore suggest that histamine release is not responsible for the respiratory effect of intravenously injected 5-HT.

2. *Anticholinergic drugs:* To determine whether the respiratory effects noted were of central origin or were mediated through vagal fibres, atropine in doses of 1.2 mg. were given intravenously to several normal subjects. As illustrated in Fig. 3, the respiratory effect of 5-HT was not abolished, although it was somewhat altered. This result appears to be in keeping with

that obtained by Douglas and Toh[11] who performed vagotomies in animal studies. However, despite the lack of complete reproduction of the 5-HT effect after atropine, the systemic complaints and the subjective effects were not altered in any degree.

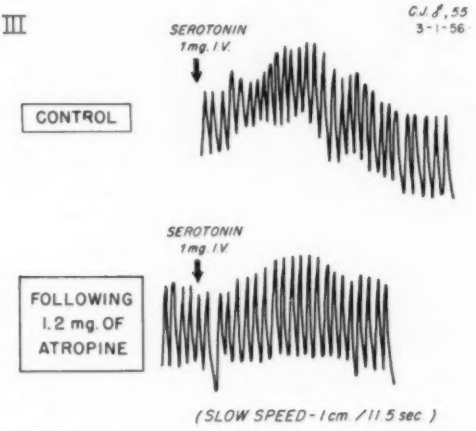

Fig. 3.—Effect of atropine on the respiratory response to serotonin.

Six patients with hypertensive cardiovascular disease who were receiving large doses of hexamethonium chloride were studied. Four patients with hypertensive cardiovascular disease were given hexamethonium chloride intravenously in doses producing a prompt lowering of the blood pressure. 5-Hydroxytryptamine was given intravenously to both groups at a time when the hexamethonium chloride was active in lowering the blood pressure. Again no significant interference by hexamethonium with the respiratory effects of 5-HT was evident.

Phentolamine (Regitine) and chlorpromazine in 5 mg. and 25 mg. doses given intravenously were likewise ineffective in preventing the respiratory response.[12]

3. *Benzyl analogue of 5-HT:* This drug, 1-benzyl, 2,5-dimethyl serotonin (BAS), has been shown to block the blood pressure response to 5-HT in both animals and man. No effect from BAS itself was noted.[13]

Seven normal and 7 asthmatic patients were premedicated in the recumbent position with 50 to 150 mg. of BAS given intravenously without producing subjective sensations. Partial to complete inhibition of the respiratory effects of intravenously injected 5-HT occurred in each instance and the systemic side effects were also strikingly inhibited. Fig. 4 illustrates complete inhibition. This could be overcome either by increasing the dose of 5-HT or by decreasing the dose of BAS.

Our studies with atropine, hexamethonium, and pyrilamine maleate would appear to confirm those of Heymans,[14] who concluded that the respiratory effect in animals was due to stimulation of the central nervous system rather than action on peripheral nerves as suggested by Douglas and Toh.[11]

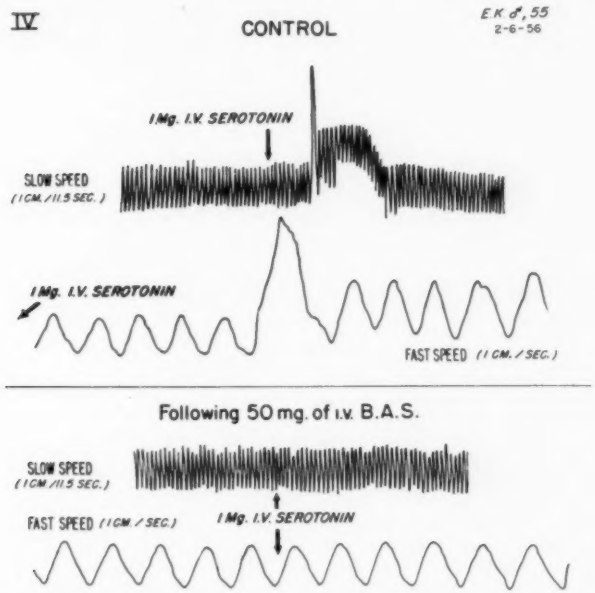

Fig. 4.—Effect of the benzyl analogue of serotonin on the respiratory response to serotonin.

DISCUSSION

Our studies indicate that 5-hydroxytryptamine (5-HT) injected intravenously produces consistent respiratory effects which are probably the same in both normal and asthmatic subjects. The respiratory effects are usually stimulatory but sometimes inhibitory depending on the individual studied. Bronchial obstruction was not induced, an unexpected finding in view of studies in guinea pigs[15] and the occurrence of wheezing in certain patients suffering from malignant carcinoid.[1-3] Whether these patients had underlying obstructive pulmonary disease with intensification of symptoms secondary to the serotonin effect, is unknown. However, studies to be described in a subsequent article, showed that aerosolized 5-HT given to asthmatic subjects produced a prompt fall in vital capacity and decrease in expiratory flow rate.[16] One may speculate that the doses of 5-HT used here, even though they evoked an intense physiologic response, may be less than the amounts reaching the lung in certain clinical states. Possibly higher dosage of 5-HT reaching the lung by way of the circulation might provoke bronchial obstruction. Our studies thus far, indicate that in its effect on respiration,

5-HT given by intravenous injection, acts on specific receptor sites in both normal and asthmatic subjects. BAS (anti-serotonin) presumably acts by competing with 5-HT for these specific receptors.[17]

SUMMARY

1. The effect of intravenously injected 5-hydroxytryptamine (serotonin) on respiration in 22 nonasthmatic and in 8 asthmatic subjects was studied. Hyperpnea and tachypnea was noted on 27 occasions and in 3 normal subjects transient apnea occurred, followed by hyperpnea.

2. The respiratory response was potentiated by pyrilamine maleate given intravenously.

3. The respiratory effect was unaltered after anticholinergic drugs, ganglionic, and adrenegic blockade but was inhibited by the benzyl analogue of 5-hydroxytryptamine.

4. The available evidence in man suggests that the respiratory effect of intravenously injected 5-hydroxytryptamine is mediated through action on specific receptors.

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THE ELASTIC PROPERTIES OF THE LUNGS IN THE AGED

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CHANGES in the lung compartments are known to occur with aging. The vital capacity diminishes while the residual volume enlarges. Pulmonary ventilation at rest is not greatly altered and normal amounts of oxygen and carbon dioxide are found on analysis of arterial blood. Maximum ventilation is compromised, but tolerance for ordinary exercise is preserved.[1] Individual studies have demonstrated a diminished pulmonary diffusing capacity[2] and an uneven distribution of inspired gas.[3]

Measurements of the elastic properties of the lungs and the mechanics of pulmonary ventilation have not previously been reported. They are essential for an improved understanding of the physiology of aging, and may also be of clinical importance. It is the purpose here to present such measurements on a group of elderly subjects.

SUBJECTS

It was considered essential to obtain a random selection of subjects. A relationship between the occurrence of prostatic hypertrophy and diseases of the lungs is not known. Thus, all white men admitted to the hospital for prostatic resection during the period July 15, 1956, to November 15, 1956, were studied. It was required only that they be 65 years or older. The attending surgeon controlled admissions without altering his routine policies for providing patient care. Four of 19 such subjects are not included since they failed to complete all procedures. The reasons are: one died, one was psychotic and disoriented, and two refused to cooperate fully. The study group is consecutive except for these unavoidable lapses. Healthy young individuals served as a control group for the compliance measurements. They were interns and medical students whose ages ranged from 20 to 27 years.

None of the study group subjects had pulmonary symptoms as major complaints. Two subjects (5 and 8)* had a mild chronic cough and produced small amounts of sputum. One subject (12) had dyspnea on exertion which he considered significant, while 5 other subjects (2, 3, 5, 9, and 10) who reported this symptom did not believe it was abnormal in amount. Two subjects (8 and 13) had had asthma in the past, but neither had experienced an asthmatic attack for more than one year prior to study.

Two subjects (11 and 12) had a typical barrel chest deformity. Seven (1, 8, 9, 10, 12, 13, and 14) exhibited hyperresonance to percussion of the chest. Auscultation did not reveal a significant number of râles in any subject.

METHODS

All spirometric measurements and residual volume determinations were performed with the patient sitting upright. Gas volumes were corrected to 37° C. saturated with

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*Numbers in parentheses refer to case numbers listed in the tables.

water vapor. Residual volume measurements were by the method of Darling and co-workers.[4] The expiratory reserve volume was measured immediately prior to each determination to eliminate the effect of variation in the level of pulmonary mid-capacity. The average differences in duplicate nitrogen analyses were 0.02 and 0.04 vol. per cent for the gasometer and alveolar air samples, respectively. Duplicate residual volume determinations on the 15 subjects agreed to an average of 91 ml. or 3.2 per cent. Determinations performed on normal individuals and patients with congestive heart failure during the study period served as a constant check on the accuracy of the procedure. Maximum breathing capacity was determined with a 15-second effort. Gas collected in a neoprene balloon through a low-resistance valve was measured in a Tissot gasometer.

Arterial blood was obtained anaerobically with the subject at rest in the supine position. Analyses for carbon dioxide and oxygen content were by the method of Van Slyke and Neill.[5] The average difference in duplicate analyses was 0.05 vol. per cent for oxygen. Hemoglobin oxygen capacity was determined with the Sendroy chamber equilibration method.[6] Duplicate checks averaged 0.11 vol. per cent. Hemoglobin saturation was calculated by subtracting dissolved oxygen (0.3 vol. per cent) from the content, and dividing by the capacity. It has been multiplied by 100 for expression as per cent. Blood pH was measured anaerobically at room temperature with a glass electrode in a Beckman model Gs pH meter. Correction for temperature was made.[7] The nomogram of Singer and Hastings[8] was used to estimate pCO_2.

Static pulmonary compliance was measured by the intraesophageal balloon technique described by Stead, Fry, and Ebert.[9] Pulmonary mid-capacity was determined directly from the spirometer tracing of the compliance study in every instance. Mean compliance curves were plotted by averaging several determinations for each point. Tangents to the curve at various levels of lung inflation yielded the final measure of compliance. Functional compliance in the first 4 subjects, was recorded in a manner similar to that described by Mead and Whittenberger[10] from the face of a cathode ray tube. Instead of using a double coated lens, however, "loops" were traced directly on a plastic screen. Functional compliance in all other subjects was obtained using a Sanborn Twin-Viso recorder. The pneumotachograph was calibrated during each procedure. Its tracing was integrated for volume by counting squares. Measurements of airway resistance were performed at pulmonary mid-capacity, by the method of Fry, Ebert, Stead, and Brown.[11]

TABLE I. PHYSICAL CHARACTERISTICS AND PULMONARY FUNCTION STUDIES IN A GROUP OF AGED SUBJECTS

SUBJECT NO.	AGE (YR.)	BODY SURFACE AREA ($M.^2$)	TOTAL LUNG CAPACITY (ML.)	VITAL CAPACITY (ML.)	RATIO RV/TLC* (%)	FUNCTIONAL RESIDUAL CAPACITY (ML.)	INDEX OF MIXING (%)	MAXIMUM BREATHING CAPACITY (L./MIN.)	pCO_2 (MM. HG)	ARTERIAL OXYGEN SATURATION (%)
1	70	1.69	7,825	4,434	43.3	5,747	2.935	65.6	46	95.6
2	65	1.97	7,564	4,545	39.9	4,915	2.401	50.3	31	90.5
3	71	1.84	6,370	3,661	42.5	4,019	1.231	41.1	37	93.0
4	68	1.87	5,333	3,538	33.7	3,043	0.570	64.5	30	90.3
5	69	1.74	7,565	3,130	58.6	6,063	5.588	24.6	38	94.5
6	75	1.79	7,449	4,495	39.6	4,967	0.917	118.4	38	93.1
7	77	1.72	5,314	2,519	52.6	4,235	0.649	58.8	39	97.5
8	68	1.81	6,641	4,056	38.9	4,737	1.321	73.9	41	93.4
9	75	1.44	6,818	2,800	58.9	5,560	4.123	28.1	39	94.6
10	69	1.68	6,423	3,680	42.7	4,958	1.866	56.3	31	95.1
11	76	1.74	6,297	3,577	43.2	4,289	0.832	96.1	37	94.1
12	86	1.82	5,883	3,124	46.9	4,341	1.923	57.8	38	95.3
13	70	1.90	7,569	3,558	53.0	5,135	1.533	33.6	40	94.7
14	65	1.69	5,988	3,185	46.8	3,727	1.999	65.9	33	95.7
15	65	1.68	5,694	3,671	35.5	3,812	0.419	67.1	37	97.4
Mean	71	1.76	6,582	3.598	45.1	4,637	1.887	60.1	37	94.3

*Residual volume/total lung capacity.

RESULTS

Individual values are shown in Tables I and II. Fig. 1 presents the frequency distribution of the routine physiologic studies. The total lung capacity averaged 6.6 L. The mean functional residual capacity was 4.6 L. The concentration of nitrogen remaining in alveolar air at the end of 7 minutes oxygen

TABLE II. PULMONARY COMPLIANCE AND AIRWAY RESISTANCE MEASUREMENTS IN A GROUP OF AGED SUBJECTS

SUBJECT NO.	COMPLIANCE STATIC (L./CM. H_2O)	COMPLIANCE FUNCTIONAL (L./CM. H_2O)	COMPLIANCE RAPID RATE FUNCTIONAL (L./CM. H_2O)	LINEAR COEFFICIENT	QUADRATIC COEFFICIENT
1	0.500	0.391	--	0.29	0.39
2	0.667	0.223	--	1.47	0.67
3	0.443	0.183	--	1.73	0.37
4	0.396	0.150	--	1.34	0.27
5	0.333	0.195	0.129	3.30	5.27
6	0.500	0.227	0.158	0.47	0.12
7	0.247	0.113	0.135	1.09	0.26
8	0.267	0.118	0.101	1.03	0.19
9	0.272	0.105	0.088	1.23	1.21
10	0.328	0.195	0.174	1.07	0.33
11	0.500	0.219	0.202	1.21	0.12
12	0.303	0.144	0.106	1.30	1.35
13	0.384	0.241	0.156	2.70	4.80
14	0.208	0.096	0.092	1.05	0.80
15	0.417	0.261	0.224	0.67	0.21
Mean	0.384	0.191	0.142	1.33	1.09

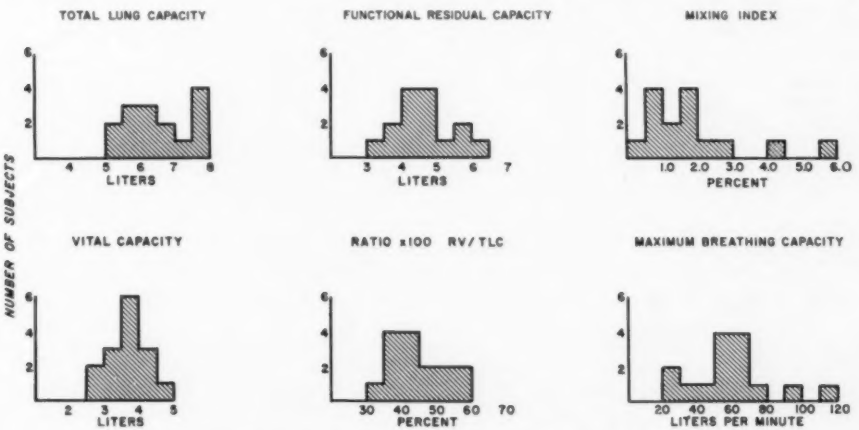

Fig. 1.—Frequency distribution of pulmonary function studies in aged subjects.

breathing was less than 2.5 per cent in 12 of the 15 subjects. The mean vital capacity was 3.6 L. The average ratio of residual volume to total lung capacity was 45.1 per cent. The maximum breathing capacity varied from 24 to 118 L. per minute, the mean being 60 L. per minute. These values are in agreement

with means reported in the literature for healthy aged subjects.[3, 12] The functional residual capacity and the ratio of residual volume to total lung capacity are greater in aged individuals than in young persons.

Fig. 2 compares the pressure volume diagram of the lungs of a subject in the aged group with a healthy young person. The total lung capacities are comparable. The aged subject has a larger residual volume and functional residual capacity than the young individual. Also, the entire pressure volume curve is shifted to the left in the aged subject reflecting less negative intrathoracic pressure.

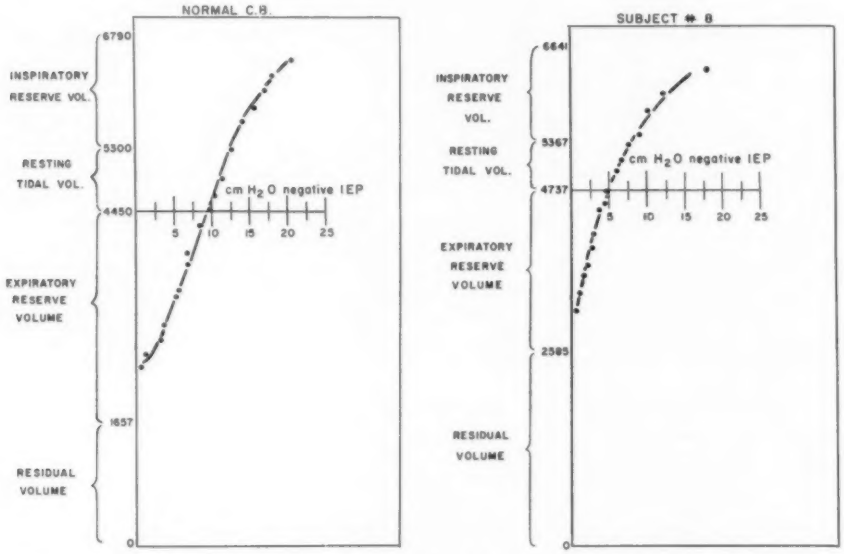

Fig. 2.—Pressure volume diagrams of the lungs. A healthy 28-year-old intern (on the left) is compared to a 68-year-old subject (on the right).

Table III shows the variation in compliance encountered over the range one liter on either side of pulmonary mid-capacity. The marked change seen in the study group contrasts sharply with the constant values observed in the

TABLE III. COMPARISON OF MEAN VALUES FOR STATIC COMPLIANCE BETWEEN AGED SUBJECTS AND HEALTHY YOUNG INDIVIDUALS*

	STATIC COMPLIANCE			
	SUBJECTS		NORMALS	
	NO.	COMPLIANCE (L./CM. H_2O)	NO.	COMPLIANCE (L./CM. H_2O)
1 L. above	14	0.185	8	0.239
0.5 above	15	0.246	8	0.250
Mid-capacity	15	0.350	8	0.245
0.5 below	15	0.455	8	0.238
1 L. below	10	0.625	6	0.239

*Compliance in this table is the reciprocal of the mean elastance. It differs slightly from the true mean compliance (shown in Table II).

young control group. This may be better illustrated in Fig. 3 where the curvilinear nature of the pressure volume diagram obtained in the aged subjects is readily apparent.

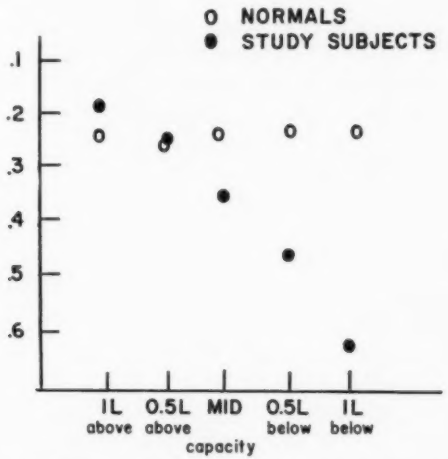

Fig. 3.—Mean static compliance in aged study subjects and normal young individuals. The aged group is represented by the black dots. These points correspond to the figures shown in Table III.

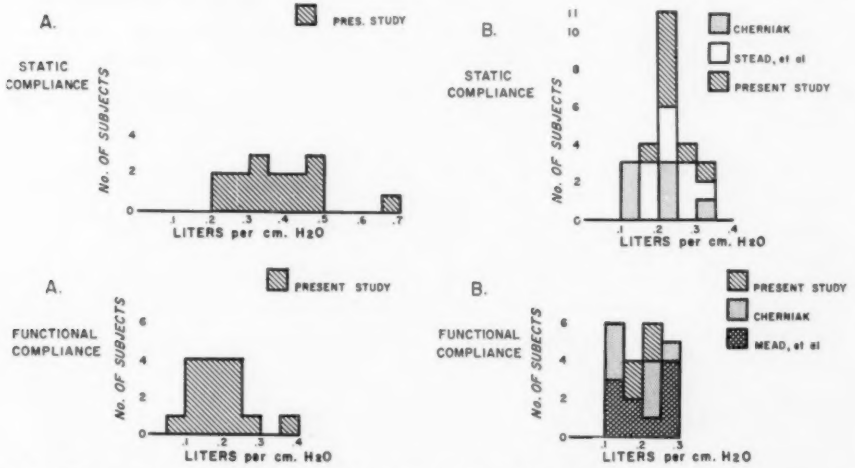

Fig. 4.—Frequency distribution of static and resting functional compliance measurements. The present group of aged subjects is shown in the left hand figures. The right hand figures represent healthy young individuals from the present study and collected from the literature. See Stead and associates,[9] Mead and associates,[10] and Cherniack.[12]

Fig. 4 compares the frequency distribution of the compliance measurements at the functional residual capacity in the present study group with healthy young subjects measured in this laboratory and collected from the

literature.[9, 13, 14] The static compliance of the aged subjects ranges from 0.21 to 0.67 L. per cm. H_2O, with a mean of 0.384. The young subjects range from 0.12 to 0.33 L. per cm. H_2O, with a mean of 0.233. A mean static compliance of 0.19 L. per cm. H_2O was found in a recent study of 38 normal young men.[15] The resting functional compliance of 13 of the 15 aged subjects lies between the levels of 0.1 and 0.3 L. per cm. H_2O where all of the young subjects are located. Thus a difference exists between the groups in static compliance, but not in resting functional compliance.

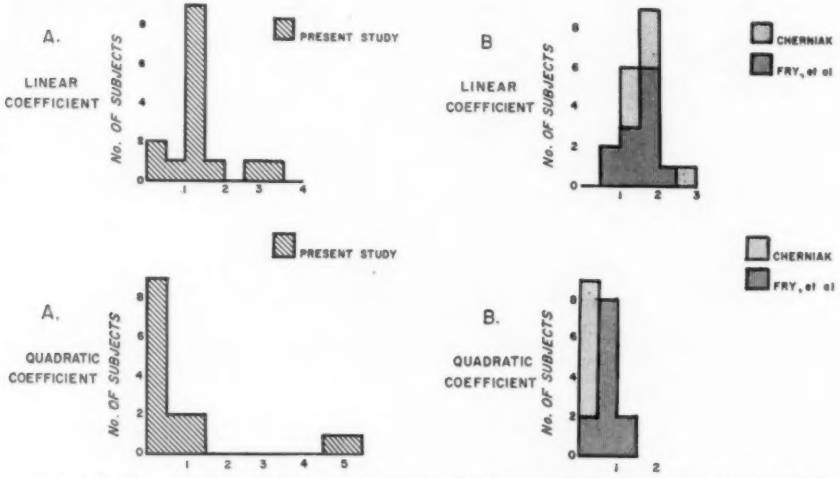

Fig. 5.—Frequency distribution of airway resistance measurements. The 15 aged subjects are depicted in the left hand figures. The right hand figures are compiled from the data of Fry and co-workers[11] and Cherniack[13] for healthy young subjects.

Airway resistance measurements in the aged subjects are compared to healthy young subjects collected from the literature[11, 13] in the frequency distribution curves shown in Fig. 5. The peak incidence of the linear coefficient occurs between 1.0 and 2.0 in each group. The range 0 to 1.5 which contains all of the values for quadratic coefficient in the young group, is seen to contain 13 of the 15 aged subjects. It is apparent here that two aged individuals differ markedly from the remainder of the group. These subjects may belong to a different population group and may have obstructive pulmonary emphysema. In general, there is no significant difference in the airway resistance of the aged subjects as compared to healthy young individuals.

DISCUSSION

A characteristic finding in aged individuals as compared with normal young persons is an increase in residual volume and functional residual capacity, and a decrease in vital capacity. It seems logical that these changes in the lung compartments result from a change in the elastic properties of the lungs with aging. Evidence favoring this hypothesis was obtained in the present study where marked changes in the elastic properties of the lungs of the aged individuals were found.

The intrathoracic pressure was less negative in the aged than in the young normal subjects at any given level of lung inflation. This can only result from an alteration in the elastic properties of the lungs. Whether this change is due to a reduction in the number of elastic fibers, alterations of the elastic properties of existing fibers, or a combination of these factors has not yet been determined. A striking feature of the pressure volume diagram in the aged subjects is its curvilinear form. The reason for this contour is not clear, but a similar type of curve may be observed in patients with pulmonary emphysema. This differs from the relatively linear diagram found in healthy young subjects (see Fig. 3).

Functional compliance is determined by simultaneously registering changes in pulmonary volume and intrathoracic pressure during continuous breathing. The assumption is made that the pressure recorded at the instant of zero air flow relates only to the elastic properties of the system. Several observers[10, 13] have reported close agreement between resting functional and static compliance measurements in healthy young subjects. Moreover, functional compliance in these subjects is not altered by marked changes in the rate of breathing. Otis and associates[16] have demonstrated that multiple air pathways with equal time constants (resistance times compliance equals time constant) should behave in this manner.

Marked discrepancy occurred in the measurements of functional and static compliance in the present group of aged subjects. This suggests that the compliance of various portions of the aged lung may not be the same, and hence the time constants for different pathways may vary. If this were true, there would be a phase lag between the various respiratory units.[16] In the presence of such a phase lag, the initial assumption of static conditions at the point of zero flow in the trachea is invalid. Air may be moving into some alveoli and out of others. Under these circumstances, the concept of functional compliance becomes meaningless.

The majority of the aged subjects were found to have normal airway resistance in contrast to patients with obstructive pulmonary emphysema in whom the resistance is regularly increased. It should be pointed out that the maximum rate of expiratory air flow is influenced by the elastic properties of the lungs.[11] The less negative intrathoracic pressure found in aged individuals results in a decrease in the maximum rate of air flow as compared with young persons because of the check valve mechanism described by Dayman.[17]

The validity of the measurements of compliance and resistance rests on the accuracy of the intraesophageal pressure measurement as an index of intrathoracic pressure. Comparisons of intrapleural and intraesophageal pressure would indicate that intraesophageal pressure can be used as a valid measure of change in intrathoracic pressure.[18] The absolute measurements of intraesophageal and intrapleural pressures differ somewhat, intraesophageal pressure tending to be more positive. In spite of this, it seems likely that very little error will occur when the means of large groups of individuals are compared.

It is of interest that the changes in the lung compartments and over-all elastic properties of the lung are similar in aged individuals and patients with emphysema. The difference between these two groups lies in the increased resistance to air flow and the abnormal distribution of inspired gas in emphysema. Even here, the distinction is somewhat arbitrary. One elderly subject was found to have abnormalities in both airway resistance and alveolar nitrogen concentration after breathing oxygen for 7 minutes. One other subject had only abnormal airway resistance while two others had only abnormal alveolar nitrogen concentrations after breathing oxygen. Studies with a technique more sensitive than the one used here, have revealed slight abnormalities in the distribution of inspired gas in a majority of healthy aged males.[3] While these changes were not of the magnitude usually seen in emphysema, they were quite definite. It is apparent that although clear-cut differences exist between groups of individuals, it may be extremely difficult in any given aged individual to differentiate emphysema with certainty. None of the present physiologic measures permits the categorical diagnosis of emphysema in every instance.

SUMMARY

1. Physiologic studies commonly employed to evaluate pulmonary function were performed in 15 unselected white men over 65 years of age. Compliance of the lung and airway resistance were also measured.

2. An increased residual volume, an increased functional residual capacity and a decreased vital capacity were demonstrated in the aged subjects as compared to healthy young individuals.

3. Marked changes were found in the over-all elastic properties of the lungs of the aged subjects. The static intrathoracic pressure was less negative for any given level of lung inflation and the pressure volume diagram was curvilinear.

4. Measurements of airway resistance were normal in the majority of the aged subjects. This finding sharply differentiates these subjects from patients with obstructive pulmonary emphysema.

5. It is suggested that aging produces a primary change in the elastic properties of the lungs which is accompanied by marked alteration of the pressure volume diagram of the lungs and increase in the residual volume. The vital capacity is compromised as a result of these changes.

We wish to express gratitude for the valuable help of Mr. Joe Bill Hocott.

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CARDIAC OUTPUT AND RELATED MEASUREMENTS AND PRESSURE VALUES IN THE RIGHT HEART AND ASSOCIATED VESSELS, TOGETHER WITH AN ANALYSIS OF THE HEMODYNAMIC RESPONSE TO THE INHALATION OF HIGH OXYGEN MIXTURES IN HEALTHY SUBJECTS

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SINCE the introduction of the technique of cardiac catheterization in man for the measurement of right heart pressures and the calculation of cardiac output by the Fick principle,[1] various reports have appeared in the literature defining the normal range of pressures in the right heart, pulmonary vessels, and great veins.[2-13] The number of subjects used for study has, however, been small in most cases and the data frequently have been incomplete. It is the purpose of the present communication to present a detailed analysis of pressures in the various right heart chambers and great vessels together with calculations of cardiac output, vascular resistances and related data in an adequate series of healthy ambulatory subjects. In addition the effects of inhalation of high oxygen mixtures will be analyzed. The blood oxygen saturation values in the right heart and great vessels in these same subjects will be reported separately.[14] During the present procedure 12 subjects were exercised in the supine position. The data obtained in this connection will also be reported separately.[15] The subject numbers used in all three publications are identical.

MATERIAL AND METHODS

Of the 26 normal subjects 15 were healthy male physicians, aged 27 to 42 years, and the remaining 11 (3 female, 8 male, aged 13 to 44 years) were patients who had cardiac catheterization performed to exclude cardiac abnormalities (Table I). The physicians were volunteers considered clinically to have completely normal cardiovascular systems, so that in 3 of these subjects no attempt was made to pass the catheter into the pulmonary artery or pulmonary artery wedge position or to obtain complete flow data with the subject breathing room air. In all 26 subjects multiple dye-dilution curves were recorded[16] following the injection of Evans blue dye at various central sites[17] and found to be normal. Subject 7 was excluded from Tables II through IX because the zero level for pressure measurements was unsatisfactory. This subject has, however, been included in the report analyzing blood oxygen saturation values.

Cardiac catheterization was performed according to the method of Cournand and Ranges[1] as modified by Wood.[18, 19] The subjects had a light breakfast and received 0.5 grain of codeine and 1.5 grains of seconal 15 to 30 minutes before the procedure commenced. They lay supine with the table horizontal. Room temperature was kept constant at 72° F. The

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oral temperature was normal in all subjects. In all except Subjects 1, 5, and 24, a No. 6 Cournand-type catheter with a bird's-eye tip was inserted by needle introduced percutaneously[19] into a right antecubital vein and advanced into the heart under fluoroscopic control. A No. 5 catheter was used in Subjects 1 and 24 and a No. 7 catheter in Subject 5. No difficulty was encountered in advancing the catheter tip into the pulmonary artery or pulmonary artery wedge position when this was attempted, but in four cases the inferior vena cava was not entered.

Pressures were recorded from the venous catheter at a sensitivity of 2 mm. deflection per millimeter of mercury, and from a needle indwelling in the right radial artery, by strain-gauge manometers[20, 21] suitably damped.[19, 22] Oxygen saturations were measured intermittently by whole blood cuvette oximeters[23] attached by three-way stopcocks to the venous catheter and arterial needle and continuously by earpiece oximeters[24] attached to one or both ears. The catheter-cuvette system was intermittently flushed clear of blood by means of a wash bottle containing 0.9 per cent sodium chloride solution with 20 mg. of sodium heparin per liter at a pressure of 200 to 300 mm. Hg. Heart rate was recorded by an instantaneous cardiotachometer,[25] and the respirations were recorded by a thermocouple attached inside a plastic mould placed over the subject's nose and mouth. When using a mouthpiece (that is, during the oxygen-uptake studies and while the subject was breathing oxygen) respirations were recorded by measurement of the pressure changes within the mouthpiece using a sensitive strain-gauge manometer.[18] The electrocardiogram, recorded from transverse chest leads, was made continuously visible to the operator (together with the venous pulse contour) by the use of twin cathode-ray tubes with long-persistence screens.[19] In this way cardiac irregularities were immediately detectable. Intrapleural pressures were not measured. A continuous record of these physiologic variables was obtained at paper speeds of 1.25 or 5 mm. per second, throughout the procedure by a photo-oscillographic assembly described elsewhere.[18] A second camera was used for simultaneous recordings at paper speeds of 25 or 150 mm. per second at intervals during the procedure.

Repeated measurements of maximal and minimal (systolic and diastolic) pressures were made from the pressure tracings of antecubital vein (ACV), superior vena cava (SVC), inferior vena cava (IVC), right ventricle (RV), pulmonary artery (PA), pulmonary artery wedge positions, and systemic (radial) artery over a minimum of two complete respiratory cycles and were averaged for inspiration and expiration. The average of the inspiration and expiration values was taken as the over-all average maximal or minimal pressure. Although not a true average, the respiratory phases being unequal, this pressure was considered satisfactory for present purposes. All pressure pulses were measured by the one observer to eliminate as far as possible varying interpretations of the motion artifact[26] present in the different tracings, particularly those from the RV and PA.

Right ventricular diastolic pressures were measured at two points (Fig. 1) called initial diastolic (RV_{di}), corresponding to the sharp downswing on the pulse tracing in early diastole following opening of the tricuspid valve, and end diastolic (RV_{de}), the point prior to the upswing of systolic pressure. The pulse pressure was the difference between the end diastolic and maximal systolic pressures. The pulmonary artery wedge maximal pressure in these subjects was usually the "v" wave, the minimal pressure being taken as the lowest point of the tracing (Fig. 1). The methods used in measuring the remaining pulse contours are also shown in Fig. 1. Mean pressures were obtained by planimetric integration of the area below the pulse wave over two complete respiratory cycles.

The reference point for all pressures was taken as midway between the sternal end of the third intercostal space and the back (or table top) and has been called "mid-chest" level. Repeated pressure calibrations were made throughout the procedure and at its conclusion, a mercury manometer being used as the standard of reference. At the termination of the procedure the mid-chest level was checked by taking the midpoint between the pressure tracings obtained with the saline-filled catheter tip placed first on the third anterior intercostal space at the sternum and secondly at table-top level.

With the subject at rest breathing room air, oxygen-uptake studies were performed by the collection of expired air, using a mouthpiece and nose clip, in a Tissot spirometer

over a 4- to 7-minute period (average 6 minutes). The sample was analyzed for oxygen and carbon dioxide content in the Haldane apparatus. During the middle of the oxygen-uptake studies, simultaneous blood samples were collected from the pulmonary artery and radial artery and analyzed within 30 minutes for oxygen content by the method of Van Slyke and Neill.[27] Oxygen capacity was obtained after equilibration by the method of Sendroy[28] as modified by Roughton and associates.[29] Midway during withdrawal of the blood samples for manometric analysis flow was stopped while a recording, at a paper speed of 150 mm. per second, was made of pressures in the radial and pulmonary arteries.

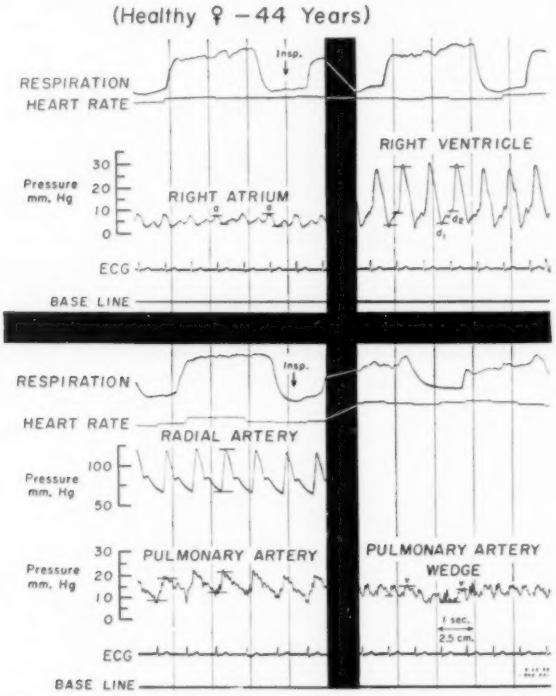

Fig. 1.—Pulse contours at various sites (Subject 26) recorded at a paper speed of 25 mm. per second. The points at which maximal and minimal pressures were measured are shown for each pulse contour. Right atrial maximal pressure was usually the "a" wave. Right ventricular diastolic pressure was measured at two points (d_1 and d_2). The pulmonary artery wedge maximal pressure was usually the "v" wave.

In all subjects, except Number 25, oxygen was administered throughout the latter part of the procedure by a closed-circuit spirometer system with an approximate capacity of 80 liters, using a mouthpiece and nose clip. The system was filled with 100 per cent oxygen which, after equilibration with the respiratory gases, resulted in a mixture of approximately 95 per cent oxygen and 5 per cent nitrogen. The duration of oxygen administration averaged 78 minutes and ranged from 39 to 208 minutes. Recordings of the pressure pulses from the radial and pulmonary arteries were made at a paper speed of 25 mm. per second, immediately before the subject commenced breathing oxygen and again 3 to 5 minutes later, the catheter position remaining constant during this interval. From 6 to 33 (average 16) minutes after administration of oxygen had been begun, blood samples were withdrawn simultaneously from these vessels for Van Slyke analysis. The average slope of the curve of oxygen consumption was measured over an 8-minute period centered on the time during which the blood samples were withdrawn simultaneously from the radial and pulmonary arteries.

The following calculations were made while the subjects were breathing air and oxygen*[30]:

$$1.\ \dot{Q} = \frac{\dot{V}_{O_2}}{C_aO_2 - C\bar{v}O_2},\qquad 2.\ R_s = (60P\bar{s}/\dot{Q}) \times 1.332,$$

$$3.\ R_p = (60P\bar{p}/\dot{Q}) \times 1.332,\qquad 4.\ R_{pa} = [60(P\bar{p} - P\overline{w})/\dot{Q}] \times 1.332.$$

*$\dot{Q}$ = cardiac output (liters/minute); $\dot{V}_{O_2}$ = oxygen consumption (milliliters/minute STPD); C_aO_2 = oxygen content of systemic arterial blood (milliliters/liter); $C\bar{v}O_2$ = oxygen content of mixed venous blood from the pulmonary artery or right ventricular outflow tract (milliliters/liter). R_s = "total" systemic vascular resistance (dynes·seconds·cm.$^{-5}$); R_p = "total" pulmonary vascular resistance (dynes·seconds·cm.$^{-5}$); R_{pa} = pulmonary "arteriolar" vascular resistance (dynes·seconds·cm.$^{-5}$); $P\bar{s}$ = systemic (radial) artery mean pressure (mm. Hg); $P\bar{p}$ = pulmonary artery mean pressure (mm. Hg); $P\overline{w}$ = pulmonary artery wedge mean pressure (mm. Hg). $P\bar{s}$ and $P\bar{p}$ were measured midway during withdrawal of the blood samples for Van Slyke analysis or within a few moments of the latter. $P\overline{w}$ was measured as close as possible to the withdrawal of the blood samples, the interval varying from 5 to 30 minutes.

RESULTS

Cardiac Output and Related Measurements.—The data concerning cardiac output and vascular resistances in these 26 subjects while breathing air are detailed in Table I, together with other related measurements. Chest thickness is included to make possible comparison of the pressure data with other series in which different zero reference levels have been selected. The mean metabolic rate of +4 per cent was within the normal basal range. The mean respiratory quotient of 0.82 suggested that the subjects were in a steady state, although there were abnormalities in both these values in Subjects 4 and 16. Because the cardiac output in Subject 4 was far in excess of all other subjects (cardiac index 6.3 L./min./M.^{2}), the data concerning flow and resistance in this subject have been excluded from the calculations of means and standard deviations. The oxygen capacity of blood was normal in all cases with a mean of 19.7 volume per cent. Oxygen consumption averaged 143 c.c./min./M.^{2}, which compares well with other estimates on normal subjects.[31] The mean cardiac index equaled 3.5 L./min./M.^{2} (range 2.5 to 5.3) and the stroke index 46 c.c./beat/M.^{2} (range 37 to 63). The corresponding values for cardiac output and stroke volume were 6.6 L./min. (4.4 to 8.9) and 96.3 c.c./beat (63.1 to 122.0), respectively. These figures are comparable to those given by others.[7, 10, 32-35] The "total" pulmonary resistance of 205 dynes·seconds·cm.$^{-5}$ was approximately one-sixth the total systemic resistance of 1,130 dynes·seconds·cm.$^{-5}$ The pulmonary "arteriolar" resistance averaged 67 dynes·seconds·cm.$^{-5}$ The range of resistance values was wide.

Pressures at Various Sites.—The average maximal, minimal, and mean pressures in individual subjects, expressed in millimeters of mercury, are listed in Table II and the average pulse pressures for all subjects in Table III. The several measurements of these pressures made throughout the procedure at each site were averaged for each subject to give a representative figure for pressure values during a cardiac catheterization procedure, the average pressures for all subjects and the standard deviations in both tables being calculated from these values. The ranges of pressure, however, are the ranges of individual measurements.

TABLE I. CARDIAC OUTPUT AND RELATED MEASUREMENTS

SUBJECT	AGE (YR.)	SEX	HEIGHT (IN.)	WEIGHT (LB.)	SURFACE AREA ($M.^2$)	CHEST THICKNESS (CM.)	METABOLIC RATE (%)	RESPIRATORY QUOTIENT	O_2 CAPACITY (VOL. %)
1	13	M	65	136	1.69	16	+6	0.89	17.7
2	17	M	71	170	1.97	16.5	+9	0.82	20.3
3	18	M	70	177	1.99	17.5	+5	0.86	19.1
4	19	M	69	142	1.79	20.5	+43‡	0.61‡	20.0
5	20	M	67	143	1.75	17.5	+4	0.79	21.5
6	21	M	70	158	1.89	—	−2	0.82	19.9
7	22	F	67	135	1.72	—	+18	—	18.7
8	24	M	64	145	1.71	22	+16	0.77	21.6
9	26	M	73	163	1.97	19	+3	0.83	18.2
10§	27	M	70	193	2.06	21	−7†	—	20.2†
11§	27	M	69	133	1.75	19.5	−7	0.91	19.0
12§	27	M	68	120	1.66	19.5	+17	0.73	19.0
13§	28	M	71	177	2.02	17	−3	0.66	19.3
14§	28	M	70	182	3.00	22	−7	0.75	20.9
15§	29	M	68	158	1.85	22	—	—	22.4†
16§	29	M	70	141	1.85	21	+20	1.21	20.7
17§	30	M	75	186	2.12	23	+10	0.85	18.2
18§	30	M	71	139	1.81	16.5	+14	0.80	21.2
19§	31	M	71	185	2.06	19	−7	0.76	18.8
20§	31	M	72	205	2.15	23.5	+6	0.84	21.3
21§	32	M	75	198	2.15	22	−7	0.73	20.7
22§	34	M	74	162	1.99	23	+4	0.79	19.4
23§	40	M	69	150	1.81	20	+4	0.81	18.3
24	41	F	61	119	1.52	16	−3	0.76	18.3
25§	42	M	71	185	2.05	21.5	—	—	20.5
26	44	F	66	135	1.69	17.5	+5	0.82	18.4
Mean	28		70	160	1.89	19.7	+4	0.82	19.7
S. D.			±3	±25	±0.17	±2.4	±8.2	±0.10	±1.2

*Calculated vascular resistances: R_s = "total" systemic resistance; R_{ps} = Pulmonary "arteriolar" resistance. R_p = "total" pulmonary resistance.

†Values while breathing oxygen.

‡Values excluded from calculation of mean and s.d. See text.

§Male physicians.

A slight elevation of systolic and mean systemic arterial pressures can be expected in those subjects who were not in a basal state.[36] None of the subjects were considered clinically to be hypertensive; the systolic pressures as recorded by intra-arterial needle were appreciably higher than those given by sphygmomanometer cuff. The average and range of values for systemic pressure in millimeters of mercury were: systolic 135 (106 to 164), diastolic 71 (58 to 86), mean 91 (75 to 110), and pulse pressure 64 (44 to 90).

Antecubital vein pressure was recorded once only at the beginning of the procedure in most cases. A small pulse was recognizable in all but one case, the pulse pressure varying from 0 to 3 with an average of 2 mm. Hg. Expressed as centimeters of water, the mean antecubital vein pressure equaled 11 with a range of 5 to 18.

In no case was a negative mean pressure recorded in the right atrium. A negative minimal pressure was recorded in two subjects only. The average and range of values for right atrial pressures in millimeters of mercury were: maximum 9 (3 to 14), minimum 4 (−1 to 8), mean 6 (2 to 10), and pulse pressure 5 (2 to 9).

IN 26 HEALTHY SUBJECTS WHILE BREATHING AIR

O_2 UPTAKE (C.C./MIN./M.^{2})	ARTERIO-VENOUS O_2 DIFFERENCE (VOL. %)	HEART RATE (BEATS/MIN.)	CARDIAC INDEX (L./MIN./M.^{2})	STROKE INDEX (C.C./BEAT/M.^{2})	R_v*	R_p*	R_{pa}*
					DYNES·SECONDS·CM.$^{-5}$		
166	3.8	110	4.4	40	830	160	54
167	4.2	81	4.0	49	940	240	92
154	—	89	3.8†	60†	1,140†	180†	95†
212‡	3.4	78‡	6.3‡	81‡	710‡	140‡	36‡
152	4.5	81	3.4	42	1,140	160	54
139	3.8	79	3.7	47	1,020	150	46
147	3.1	113	4.7	72	—	—	—
150	3.9	84	3.9	46	1,040	250	97
142	3.9	59	3.7	63	980	170	44
128†	4.5†	66	2.8†	46†	1,240†	290†	—
125	3.9	72	3.2	44	1,160	210	57
166	3.1	100	5.3	53	750	90	10†
137	4.1	70	3.3	47	1,170	240	84
128	4.4	75	2.9	39	1,460	230	55
—	—	86	—	—	—	—	—
157	3.7	67	4.2	63	1,000	210	82
149	5.9	62	2.5	40	1,390	—	—
143	4.9	73	2.9	40	1,130	240	106
126	3.8	80	3.3	41	1,180	220	—
144	4.3	67	3.3	49	910	200	78
127	4.5	76	2.8	37	1,340	260	52
141	3.2	75	4.4	59	780	130	18
137	4.5	80	3.0	38	1,570	220	58
118	4.1	68	2.9	43	1,540	290	91
—	—	84	—	—	—	—	—
130	4.1	84	3.1	37	1,300	240	75
143	4.1	78	3.5	46	1,130	205	67
±14.3	±0.6	±11.6	±0.7	±8.1	±178	±51	±23

The right ventricular pressure values were as follows: systolic 27 (range 16 to 36), initial diastolic 4 (range –2 to 9), end diastolic 10 (range 3 to 15), and pulse pressure 17 (range 12 to 24). The pressure difference between the initial and end diastolic levels averaged 5.5 (range 3 to 8). Examination of serial recordings of right ventricular pressure showed the initial recording of systolic pressure to be significantly higher than later recordings in those subjects in whom the catheter was passed immediately into the right ventricle at the start of the catheterization procedure. The diastolic pressure was not affected. This initial elevation of systolic pressure was presumably due mainly to the patient's early anxiety, which was aggravated by the ectopic beats often encountered when the catheter tip entered the ventricle. As the examination progressed, the subject became more relaxed. The average and range of right ventricular systolic pressure, excluding these initial recordings, were 25 (16 to 31) and the pulse pressure 16 (12 to 24).

The average values for the pulmonary artery pressure combined 16 subjects in whom the catheter lay in the main pulmonary artery and 8 with the tip in the proximal portion of the right or left pulmonary artery. Because of artifact from movement of the catheter, these tracings were difficult to measure accurately. The pressure values in the lesser circulation were approximately one-sixth the systemic and averaged 22 systolic (range 13 to 30), 12 diastolic

TABLE II. AVERAGE SYSTEMIC (RADIAL) ARTERY, PERIPHERAL VEIN, RIGHT HEART, PULMONARY ARTERY, AND WEDGE PRESSURES IN 25 HEALTHY SUBJECTS WHILE BREATHING AIR (MM. Hg)

SUBJECT	RADIAL ARTERY		ANTECUBITAL VEIN		RIGHT ATRIUM		RIGHT VENTRICLE	PULMONARY ARTERY		PULMONARY ARTERIAL WEDGE	
	PULSE	MEAN	PULSE	MEAN	PULSE	MEAN	PULSE*	PULSE	MEAN	PULSE	MEAN
1	117/61	77	13/11†	11†	9/6	8	25/6 to 11	18/12	16	12/8	10
2	147/70	92	14/11	12	14/6	9	31/7 to 13†	28/16	22	18/12	14
3	164/77	103	—	—	8/1	4	31/0 to 7	24/11	18	10/5†	8†
4	147/77	99	8/7†	7†	11/3	7	31/2 to 9	26/13	20	20/10	14
5	116/72	84	4/4	4	3/1	2	19/−1 to 4	16/ 8	12	9/6	8
6	145/67	88	6/5†	5†	5/1	3	18/1 to 6	17/ 8	12	14/7	10
8	131/71	90	9/8†	8†	10/6	8	31/7 to 11	25/16	21	16/10	13
9	156/66	88	6/3	5	8/3	5	24/3 to 8	21/11	15	14/9	11
10‡	123/68	87	14/12	13	13/8§	10	26/9 to 13	26/16	21	20/13	15
11‡	129/64	84	8/6	7	7/2	5	23/2 to 7	19/10	15	15/7	11
12‡	123/65	83	5/4	5	4/1	3	22/0 to 5	13/ 7	10	10/8†	9†
13‡	151/79	98	9/7	8	8/5	6	25/5 to 10	25/14	19	18/11	13
14‡	132/83	103	9/7†	8†	13/4	9	30/7 to 12	22/13	16	15/9	13
15‡	131/65	86	11/9	10	6/1†	4†	26/4 to 12	25/13	18	—	—
16‡	153/73	98	9/8	9	8/3	5	33/4 to 9	25/12	19	14/10	12
17‡	130/73	92	11/9	10	12/7	10	28/8 to 13	—	—	—	—
18‡	106/58	75	6/4	5	5/1	3	19/1 to 4†	21/10	15	14/5	9
19‡	140/83	100	10/8	9	10/6	8	29/7 to 13	25/16	19	—	—
20‡	122/65	84	10/7	9	10/2	5	29/2 to 8	24/12	18	12/9	11
21‡	146/76	98	10/8	9	12/6	8	28/6 to 14	26/15	21	19/13	15
22‡	120/65	83	8/6	7	7/2	4	36/6 to 13	22/10	14	15/9	12
23‡	153/86	110	10/8	9	10/3	6	30/4 to 11	21/12	17	16/9	11
24	118/68	86	7/6†	6†	8/5	6	23/6 to 10	21/12	15	13/7	10
25‡	153/80	106	—	—	11/4	7	28/4 to 11	23/12	17	—	—
26	119/67	86	11/9	10	8/3	5	27/4 to 10	20/10	16	13/9	11
Average	135/71	91	9/7	8	9/4	6	27/4 to 10	22/12	17	15/9	12
S. D.	15.7/7.4	8.7	2.8/2.5	2.5	2.9/2.2	2.3	4.3/2.7 to 2.8	3.7/2.6	3.1	2.9/2.2	2.0
Range of individual recordings											
Maximal pressure	106 → 164		4 → 14		3 → 14		16 → 36	13 → 30		8 → 23	
Minimal pressure	58 → 86		2 → 12		−1 → 8		−2 → 9*	6 → 17		5 → 14	
							3 → 15				
No. recordings	37		23		55		46	76		38	

*Diastolic values expressed as initial diastolic and end diastolic. See text.
†Values while breathing 95 per cent oxygen. Excluded from calculation of mean and s.d.
‡Male physicians.
§Corrected superior vena caval pressure substituted.

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TABLE III. AVERAGE PULSE PRESSURE AT VARIOUS SITES WHILE BREATHING AIR (MM. Hg)

	ANTECUBITAL VEIN	RIGHT ATRIUM*	RIGHT VENTRICLE†	PULMONARY ARTERY‡	WEDGE	RADIAL ARTERY
No. subjects	17	24	23	24	19	25
Average pulse pressure	2	5	17	10	6	64
Range	0 to 3	2 to 9	12 to 24	6 to 13	3 to 10	44 to 90
Standard deviation	± 0.7	± 1.9	± 3.6	± 1.9	± 1.9	± 12.4

*Superior vena cava substituted for right atrium in one subject.

†Pulse pressure measured between end diastolic and systolic levels.

‡Includes 16 subjects with catheter in main pulmonary artery and 8 with catheter in a proximal right or left pulmonary artery.

(range 6 to 17), and 17 mean (range 10 to 22). The pulse pressure averaged 10 (range 6 to 13). There was no direct relationship between pressure levels in the two circuits. A comparison was made between the average pressure in the pulmonary artery adjacent to the valve and the average in the right or left pulmonary artery in 18 subjects with tracings from both sites. The distance separating these two sites was small. A statistically significant fall peripherally in pulse pressure of 1.6 mm. Hg was noted ($P < 0.01$) but the systolic and diastolic pressure changes were inconsistent from subject to subject. The data suggested, however, that, unlike systemic arterial pressure,[37] the systolic pressure probably fell more rapidly than the diastolic as the pulse wave moved distally.

A pulmonary artery wedge pressure tracing was included when the pulse contour was considered typical with a recognizable "v" wave, whether or not a blood sample could be withdrawn. A blood sample could be withdrawn from a wedge position in 66 per cent of cases. In 88 per cent of the cases in which a blood sample was withdrawn, the sample obtained was fully saturated with oxygen (range 90 to 100 per cent). In addition to a satisfactory pulse contour, a good snap-free of the catheter tip on withdrawal from the wedge position as seen by fluoroscopy was a necessary criterion for inclusion. Following a satisfactory wedge contour and coincident with the snap-free, the pressure tracing showed the sudden appearance of gross artifact before the catheter came to rest in the proximal pulmonary artery. The average and range of pressure values for the wedge position were: maximum 15 (8 to 23), minimum 9 (5 to 14), mean 12 (8 to 15), and pulse pressure 6 (3 to 10).

Comparison of Pressures at Various Sites.—1. The differences in mean pressures between the right atrium and the associated great veins, peripheral veins, and pulmonary artery wedge position. The averages of the several mean pressures recorded in each subject were used rather than values obtained at any one particular time for, although pressure variations occurred throughout the procedure, they were found to affect the right atrium and great veins equally and in the same direction. The results are given in Table IV.

The mean pressure gradient from antecubital vein to right atrium averaged 2.3 mm. Hg. The effect of the negative intrapleural pressure was not determined. The average differences between the great veins and the right atrium indicated

a positive gradient of 0.5 mm. Hg with a range of –2 to +4. Inferior vena caval pressure measurements were confined to those in which the catheter tip lay at or above the level of the diaphragm. The finding of negative mean pressure gradients in several cases indicated that these measurements recorded one facet only of the complex pressure-flow relationship which existed at any one moment.

TABLE IV. AVERAGE MEAN VENOUS PRESSURE DIFFERENCES WHILE BREATHING AIR (MM. Hg). DIFFERENCES CALCULATED FROM RIGHT ATRIAL MEAN PRESSURE*

	ACV† MINUS RA	SVC MINUS RA	IVC MINUS RA	WEDGE MINUS RA
Number of subjects	16	21	18	19
Average difference	+2.3	+0.5	+0.5	+5.7
Range	0 to +5	−1 to +2	−2 to +2	+2 to +8
Standard deviation	±1.39	±0.75	±0.99	±1.46
P value	< 0.001	< 0.01	< 0.05	< 0.001

*+ indicates a pressure greater than RA.

†ACV = antecubital vein; RA = right atrium; SVC = superior vena cava; IVC = inferior vena cava at or above diaphragmatic level.

The differences between the mean pulmonary artery wedge and mean right atrial pressures averaged 5.7 mm. Hg with a range of 2 to 8. Since the mean wedge pressure approximates mean left atrial pressure,[38] this difference presumably gives an indication of the mean pressure gradient between the two atria.

2. The pressure gradient across the tricuspid valve. From analysis of 29 records obtained during continuous withdrawal of the cardiac catheter across the tricuspid valve, in 19 subjects, no significant pressure difference was found to exist between the maximal right atrial pressure ("a" wave of atrial systole) and the end diastolic right ventricular pressure ($P < 0.1$). There was considerable variation in different subjects (range –4 to +2 mm. Hg, s.d. ±1.7). Artifact recorded during diastole of the right ventricle made exact measurement difficult. There was also no significant difference between minimal right atrial and initial diastolic right ventricular pressures ($P < 0.4$). A comparison of the mean right atrial pressure with the arithmetic mean of the initial and end diastolic right ventricular pressures revealed a negative gradient of 1 mm. Hg from right atrium to right ventricle, which, while of statistical significance ($P < 0.01$), was of uncertain physiologic significance. A further analysis of the effect of inspiration and expiration on these differences did not reveal any statistically significant changes during the respiratory cycle. This information is not of great value, however, in the absence of simultaneous measurements of intrapleural pressure. Comparison of mean right atrial and mean right ventricular diastolic pressures in several subjects showed them to be equal. The records were not considered suitable for comparison of the mean right ventricular diastolic pressure with the mean pressure within the atrium during ventricular filling. A positive gradient from atrium to ventricle would be expected during this period.

3. The gradient across the pulmonary valve and the relation between the mean pulmonary artery pressure and mean pulmonary artery wedge pressure. These various pressure differences are detailed in Table V. The subjects were breathing air or oxygen.

The gradient across the pulmonary valve was analyzed, using either continuous records obtained during withdrawal of the catheter tip from the pulmonary trunk to the outflow tract of the right ventricle or recordings taken from these sites within 30 seconds of such withdrawal. Thirty-four records in 22 subjects were analyzed. There was a systolic gradient averaging 2 mm. Hg across the pulmonary valve which was highly significant statistically ($P < 0.001$). This difference was unrelated to the respiratory cycle. In two subjects with multiple records of this type the gradient varied from 1 to 7 and 1 to 5 mm. Hg at various times during the procedure.

Comparison of the diastolic pressures in the main pulmonary artery and in the right ventricle (Table V) revealed that at the beginning of diastole, with opening of the tricuspid valve, there was an average gradient across the pulmonary valve of 7 mm. Hg which at the end of diastole was reduced to 2 mm., a very small although statistically significant difference ($P < 0.001$).

TABLE V. DIFFERENCES (MM. Hg) BETWEEN THE AVERAGE PRESSURES IN THE PULMONARY ARTERY AND THOSE IN THE RIGHT VENTRICULAR OUTFLOW TRACT AND WEDGE POSITION. SUBJECT BREATHING AIR OR OXYGEN*

	PA SYSTOLIC MINUS RV SYSTOLIC†	PA DIASTOLIC MINUS			PA MEAN MINUS WEDGE MEAN‡
		RV INITIAL DIASTOLIC†	RV END DIASTOLIC†	WEDGE MEAN‡	
Number of subjects	22	22	22	20	20
Number of withdrawals	34	34	34	34	34
Average difference	−2.0	+6.7	+1.9	+0.1	+5.0
Range	0 to −7	+3 to +11	−2 to +6	−5 to +4	+1 to +9
Standard deviation	±1.5	±1.8	±1.6	±1.9	±1.7
P value	< 0.001	< 0.001	< 0.001	< 0.9	< 0.001

*A positive value indicates a right ventricular or wedge pressure less than pulmonary arterial pressure.

†Only records during continuous withdrawal of the catheter across the pulmonary valve (or records taken within 30 seconds of such withdrawal) are included.

‡PA = Pulmonary artery adjacent to pulmonary valve; RV = right ventricular outflow tract. Records during withdrawal from wedge to proximal pulmonary artery.

Comparison of records obtained during withdrawal of the catheter tip from the pulmonary artery wedge position to the proximal pulmonary artery (Table V) revealed no significant difference between the diastolic pulmonary artery pressure and mean wedge pressure ($P < 0.9$). The average difference between the mean pulmonary artery pressure and the mean wedge pressure was 5 mm. Hg with a range of 1 to 9.

Pressure Variations During the Respiratory Cycle.—A comparison of the average pressures during inspiration and the average during expiration at each site is presented in Table VI, expressed as differences (expiration minus inspiration). Breathing was of normal depth apart from rare periods of moderate overbreathing associated with anxiety, the effects of which were minimized by using the average differences.

Only 3 of 16 subjects showed any respiratory variation in tracings of antecubital vein pressure. In one subject only did this exceed 1 mm. Hg. The increase in both the maximal and minimal right atrial pressures during expiration was small (1 mm.) but significant ($P < 0.001$ and < 0.01, respectively).

The changes in the right ventricle were similar to those in the right atrium except for the absence of a significant change in the end diastolic pressure ($P < 0.2$), which because of artifact at the end diastolic point may have been masked.

The respiratory variations in the pulmonary artery pressure and wedge pressure were both highly significant statistically, being all in the same direction. The expiratory increase was two to three times greater than in the right atrium and right ventricle, averaging 3 mm. Hg at both sites, without any change in pulse pressure.

In the systemic arteries there was an average increase during expiration of 4 mm. Hg systolic and 2 mm. Hg diastolic with an increase in pulse pressure of 2 to 3 mm. Hg. These changes were statistically highly significant ($P < 0.001$).

The foregoing findings are essentially in agreement with the work of others.[39, 40]

TABLE VI. DIFFERENCES IN AVERAGE PRESSURES (MM. HG) DURING THE RESPIRATORY CYCLE. AVERAGE PRESSURE DURING EXPIRATION MINUS AVERAGE PRESSURE DURING INSPIRATION. SUBJECT BREATHING AIR*

	ACV	RA†	RV	PA	WEDGE	RADIAL
Number of subjects	16	24	23	24	19	22
Average difference‡	0/0	+1/1	+2/1 − 0§	+3/3	+3/3	+4/2
Range systolic differences	0 to +2	−1 to +3	0 to +4	+1 to +5	0 to +5	+2 to +9
Range diastolic differences	0 to +1	−2 to +2	−1 to +3§ −2 to +3	+1 to +5	0 to +4	0 to +5
Standard deviation systolic differences	±0.6	±1.0	±1.0	±1.2	±1.2	±1.9
Standard deviation diastolic differences	±0.3	±1.1	±1.1§ ±1.2	±1.0	±1.2	±1.1

*A positive value indicates a rise during expiration.
†Included 1 SVC and 1 IVC difference.
‡Expressed as systolic/diastolic.
§Initial diastolic and end diastolic RV pressures.

Maximal Individual Variations in Pressure During the Catheterization Procedure.—By subtracting the minimal pressure recorded at any time during the procedure from the maximal pressure, in those subjects with multiple recordings, the figures in Table VII were obtained. The diastolic pressure change was equal to approximately half the systolic, as can be seen by comparing the mean and systolic values.

The maximal systolic variations were approximately equal in the right atrium, pulmonary artery, and wedge positions, and averaged between 2 and 3 mm. Hg with a maximum of 6 mm. In the right ventricle, however, the systolic difference rose to 4 mm. Hg with a range up to 12 mm. Hg. This larger

difference could be partly explained by the high systolic right ventricular pressures recorded early in the procedure. The variation in wedge pressure was unrelated to the site of wedging of the catheter as seen fluoroscopically. Lateral chest roentgenograms were not taken.

TABLE VII. MAXIMAL INDIVIDUAL PRESSURE DIFFERENCES (MM. HG) AT VARIOUS SITES IN SUBJECTS WITH MULTIPLE RECORDINGS (AVERAGE 3, RANGE 2 TO 5). TIME INTERVAL SEPARATING DIFFERENT RECORDINGS AT ANY ONE SITE AVERAGED 75 (4 TO 305) MINUTES

	RA		RV	PA		WEDGE	
	MAXIMUM	MEAN	SYSTOLIC	SYSTOLIC	MEAN	MAXIMUM	MEAN
Number of subjects	15		13	19		9	
Average maximal differences	2.0	1.5	4.2	3.2	2.0	3.0	2.1
Range	0 to 5	0 to 4	0 to 12	1 to 5	0 to 4	0 to 6	0 to 6
Standard deviation	± 1.6	± 1.2	± 3.1	± 1.4	± 1.0	± 2.1	± 2.2

The average time difference between recordings in individual subjects at any one site was 75 minutes with a range of 4 to 305 minutes. There was no correlation between these pressure variations and the time interval separating the measurements.

The Hemodynamic Response to the Inhalation of 95 Per Cent Oxygen.— 1. Changes in heart rate, cardiac output, and vascular resistance. The simultaneous radial and pulmonary artery blood samples for calculation of cardiac output were withdrawn from 6 to 33 minutes (average 16) after commencing inhalation of oxygen. These values were subtracted from the values for cardiac output obtained from 8 to 51 minutes (average 19) before commencing inhalation of oxygen. The average time separating the measurements of cardiac output with the subject breathing air and oxygen was 35 minutes and ranged from 19 to 60 minutes. The results in 20 subjects are given in Table VIII. A positive value indicates a decrease while breathing oxygen.

There was no significant change in the oxygen capacity nor in the arteriovenous oxygen difference. The oxygen uptake was also unchanged. There was a statistically significant fall in heart rate averaging 3.4 beats per minute. Five of the 20 subjects failed to show this change and in 3 of these the increase recorded did not exceed 3 beats per minute. The average decrease in heart rate in all subjects who breathed oxygen (total 24) was 5.4 beats per minute or 6 per cent ($P < 0.01$).

No constant change in cardiac index was found, the range of differences equaling ± 0.7 L./min./m.^{2} of body surface. The stroke index, however, showed a statistically significant rise, averaging 3.0 c.c./beat/M.^{2} ($P < 0.01$). This occurred in 16 of the 20 subjects. Of the remainder a fall not exceeding 0.8 occurred in 3.

The calculated "total" pulmonary resistance decreased while breathing oxygen (Table VIII), the average fall being 23 dynes·seconds·cm.$^{-5}$ ($P < 0.01$). Only 4 subjects failed to show a decrease with differences in these cases equaling

TABLE VIII. CHANGES IN CARDIAC OUTPUT AND RELATED MEASUREMENTS PRODUCED BY BREATHING 95 PER CENT OXYGEN FOR AN AVERAGE OF 16 MINUTES, EXPRESSED AS DIFFERENCES FROM THE VALUES WHILE BREATHING AIR*

	HEART RATE (BEATS/MIN.)	OXYGEN UPTAKE (C.C./MIN./M.²)	ARTERIOVENOUS OXYGEN DIFFERENCE (VOL. %)	OXYGEN CAPACITY (VOL. %)	CARDIAC INDEX (L./MIN./M.²)	STROKE INDEX (C.C./BEAT/M.²)	R_p†	R_{ps}†	R_s†
							DYNES · SECONDS · CM.$^{-5}$		
Number of subjects	20	20	20	20	20	20	18	7	20
Average difference	+ 3.4	− 0.5	+ 0.04	+ 0.15	− 0.07	− 3.0	+ 23	− 10	− 10
Range	− 9 to + 10	− 12 to + 20	− 0.5 to + 0.5	− 0.2 to + 1.0	− 0.7 to + 0.7	− 9.7 to + 7.1	− 50 to + 89	− 46 to + 31	− 215 to + 181
Standard deviation	± 5.1	± 9.4	± 0.3	± 0.3	± 0.4	± 3.8	± 33	± 30	± 114
P value	< 0.01	< 0.9	< 0.6	< 0.1	< 0.5	< 0.01	< 0.01	< 0.4	< 0.8

*A positive value indicates a decrease while breathing oxygen.

†Calculated vascular resistances: R_p = "total" pulmonary resistance; R_{ps} = pulmonary "arteriolar" resistance; R_s = "total" systemic resistance.

TABLE IX. CHANGES IN RIGHT HEART AND SYSTEMIC PRESSURES PRODUCED BY BREATHING 95 PER CENT OXYGEN FOR A MINIMUM OF 3 MINUTES, EXPRESSED AS DIFFERENCES FROM THE VALUES WHILE BREATHING AIR (AIR MINUS OXYGEN) IN MM. HG*

	RADIAL ARTERY†				PULMONARY ARTERY†				RIGHT VENTRICLE†		RIGHT ATRIUM‡§ (MEAN)	WEDGE‡ (MEAN)
	SYSTOLIC	DIASTOLIC	MEAN	PULSE PRESSURE	SYSTOLIC	DIASTOLIC	MEAN	PULSE PRESSURE	SYSTOLIC	LATE DIASTOLIC		
Number of subjects	18	18	18	18	16	16	16	16	14	14	13	7
Average difference	− 6.0	− 3.3	− 4.2	− 2.0	+ 1.5	+ 0.6	+ 1.0	+ 0.9	+ 3.8	+ 1.7	+ 1.2	+ 2.0
Range	− 19 to + 1	− 17 to + 1	− 17 to + 3	− 7 to +5	− 3 to + 5	− 2 to + 3	− 1 to + 2	− 4 to + 5	− 2 to + 11	− 3 to + 6	− 1 to + 6	0 to + 5
S. D.	± 3.9	± 4.4	± 4.7	± 3.5	± 2.4	± 1.3	± 1.0	± 2.5	± 3.7	± 2.5	± 2.1	± 2.2
P value	< 0.001	< 0.01	< 0.01	< 0.05	< 0.05	< 0.1	< 0.01	< 0.2	< 0.01	< 0.05	< 0.1	< 0.05

*A positive value indicates a decrease while breathing oxygen.

†Compares readings taken immediately before commencing oxygen and 3 to 5 minutes later, the catheter position remaining unchanged.

‡Compares average values while breathing air and oxygen.

§Superior vena cava substituted for right atrium in 5 cases.

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0, –1, –9, and –50 dynes·seconds·cm.$^{-5}$ The change in systemic resistance was variable, although a rise was recorded in 12 of 20 cases. The pulmonary "arteriolar" resistance rose in 4 subjects and fell in 3.

2. Changes in right heart and systemic pressures. The variations in pressures associated with the inhalation of high oxygen mixtures over a period of 3 minutes or more are shown in Table IX. The systemic (radial) artery and pulmonary artery pressures recorded immediately before commencing oxygen were subtracted from those obtained after 3 to 5 minutes of oxygen administration, the position of the catheter remaining unchanged in either the main pulmonary artery or the proximal right or left pulmonary artery during this interval. In 15 subjects both pressures were recorded simultaneously. In 3 subjects only systemic and in one subject only pulmonary pressures were recorded at these times.

There was an increase in radial artery systolic pressure averaging 6 mm. Hg ($P < 0.001$), and a diastolic increase equal to approximately half the systolic ($P < 0.01$). The rise in mean pressure was thus intermediate, averaging 4 mm. Hg. The associated increase in the pulse pressure of 2 mm. Hg was slightly less constant.

The change in pulmonary artery pressure was in the opposite direction to the systemic and of lesser degree, being approximately proportional to the differences in pressure between the two circuits. The mean pulmonary artery pressure showed a decrease while breathing oxygen averaging 1 mm. Hg ($P < 0.01$). Twelve of the 16 cases showed a decrease, in two there was a rise of 1 mm. Hg and in two no change. The analysis further suggested that this change in mean pressure was due to a fall in the systolic with little alteration in the diastolic level. The failure to find a consistent decrease in pulse pressure could be a reflection of artifact present in the tracings.

Comparison between the average pressures in the right ventricular, right atrial, and wedge positions while breathing air and the average pressures at these sites while breathing oxygen was also made (Table IX). The high systolic right ventricular pressures recorded early in the procedure while breathing air were excluded. The fall in right ventricular systolic pressure while breathing oxygen averaged approximately 4 mm. Hg ($P < 0.01$). It was present in 12 of 14 subjects and in 6 cases equaled 5 mm. Hg or more. There was a fall also in end diastolic right ventricular pressure. The change in the initial diastolic level was not significant ($P < 0.8$). The average decrease in right atrial mean pressure of 1 mm. Hg was not statistically significant. The mean wedge pressure, recorded both while breathing air and oxygen in 7 subjects only, showed no change in two subjects and in the remainder a fall of up to 5 mm. Hg. There was no consistent relationship between the degree of fall in the mean wedge pressure and the mean pulmonary artery pressure in individual subjects.

COMMENT

One of the chief values of the present analysis of hemodynamic data from healthy subjects lies in its use for comparison with data obtained by similar techniques in subjects with cardiovascular disease.

The point of reference for all pressures has been taken as the midanteroposterior chest level. There can be little argument in the use of the right atrium as a suitable zero level for measurement, with reference to atmospheric pressure. Differing opinions exist, however, as to the level of the atrium within the chest, for its position will vary relative to the chest thickness of the individual. In a critical review of the subject, Lyons and associates[41] suggested that a point 10 cm. anterior to the back with the patient supine was the most accurate. These workers, although using adults showing a wide range in chest thickness, did not include children in their analysis and admitted that, in the latter case, a point 10 cm. anterior to the back would be an unsuitable zero level. Moreover analysis of their data reveals little significant difference in adult subjects between the level that they chose and the midanteroposterior chest level. Since infants and children form an appreciable number of the patients referred for cardiac catheterization, it is advisable to use a reference point suitable in both adults and children. For this reason the "mid-chest" level has been used in this laboratory. It is appreciated that an arbitrary point such as this does not give absolute accuracy, and that the levels given for right atrial and other pressures must be regarded as approximations only with reference to atmospheric pressure. With a large series of individuals, as in the present case, using the precautions previously described in establishing the exact mid-chest level, it seems justifiable to compare one subject with another and to use the present data for comparison with those collected under similar circumstances in patients with hemodynamic abnormalities.

In comparing pressure gradients, the zero level used can be ignored. A gradient of 41 mm. of water (3 mm. Hg) from peripheral vein to right atrium has been reported by Richards and associates.[2] Dexter and associates[10] reported a mean pulmonary artery pressure to mean wedge pressure gradient of 6 (3 to 9) mm. Hg. These values are similar to the present series. The pressure difference between the two atria can be estimated on assuming the wedge pressure to approximate left atrial pressure (Table IV). The mean pressure difference (wedge minus right atrial) averages 5 to 6 mm. Hg and probably is the basis for the large arteriovenous shunt present in cases of uncomplicated atrial septal defect. It is of interest that this pressure gradient is of the same magnitude as the mean gradient from pulmonary artery to pulmonary veins (Table V).

It is commonly stated that the systolic pressure gradient across the pulmonary valve is zero. This analysis, however, shows an average fall in systolic pressure of 2 mm. Hg across the valve. It has been shown in dogs[42] and man[43, 44] that the greatly increased pulmonary flow present in atrial septal defect may be associated with an appreciable pressure gradient across the pulmonary valve, which disappears with abolition of the shunt.

There is little detailed information available regarding the hemodynamic response to the inhalation of high oxygen mixtures in healthy human beings. In particular the changes occurring in right-heart pressures have not been clearly defined.

The changes in systemic arterial pressure, heart rate, cardiac output, and stroke volume during inhalation of 100 per cent oxygen have been investigated by several groups[45-48] and the results are conflicting.

Apparently the most consistent change produced by breathing 100 per cent oxygen is the fall in heart rate. This has been attributed to the rise in oxygen tension, which eliminates the normal tonic effect on the chemoreceptors exerted by the lower oxygen tension which exists while breathing air.[47] Other work[45] has suggested that peripheral vasoconstriction occurs under these conditions, which may perhaps selectively affect different organs such as the cerebral vessels. The finding in this study of a rise in systemic and a fall in pulmonary artery pressure while the cardiac output remained constant suggests that a selective change in calculated vascular resistances occurs, presumably on the basis of vasoconstriction and vasodilation. These findings are in agreement with those of Burchell, Swan, and Wood,[49] indicating that the magnitude of the left-to-right shunt increased when patients with patent ductus arteriosus and pulmonary hypertension inhaled 100 per cent oxygen. Calculations of vascular resistance in these healthy subjects do show the expected fall in "total" pulmonary resistance, but a fall in pulmonary "arteriolar" resistance with a rise in total systemic resistance was not a constant finding. It seems probable that the random errors in determinations of vascular resistance by the presently available methods prevent uniform detection of the small changes in calculated vascular resistances which presumably occur while breathing 100 per cent oxygen.

The time relations of this decrease are of interest; however, since the change in pressure is just detectable, they are difficult to determine. The decrease in pulmonary artery pressure associated with the change from breathing air to oxygen in patients with pulmonary hypertension is often dramatic, so that the time course of the effect can be studied in detail. In such patients the decrease in pulmonary artery pressure begins during the phase of rapid increase in arterial oxygen saturation approximately 10 to 20 seconds after the start of oxygen breathing and the majority of the effect is evident in 1 to 2 minutes. The present data are compatible with the belief that similar time relationships pertain to this effect of oxygen breathing in healthy subjects.

SUMMARY

Twenty-six ambulatory healthy subjects with cardiovascular respiratory systems normal in all clinical and laboratory aspects were investigated by the technique of cardiac catheterization.

The zero reference point for all pressure measurements was mid-chest at the sternal end of the third interspace.

The cardiac index averaged 3.5 and ranged from 2.5 to 4.4 L./min./M.^{2} and the stroke index 46 with a range from 37 to 63 ml./beat/M.^{2}

The mean and range of values for calculated total systemic, total pulmonary, and pulmonary "arteriolar" vascular resistances were 1,130 (745-1,570), 205 (90-290), and 67 (44-106) dynes·seconds·cm.$^{-5}$, respectively.

The average pulmonary artery wedge, pulmonary artery, right ventricular, right atrial, and antecubital vein pressures were: 15/9, 22/12, 27/4-10, 9/4, and 9/7 mm. Hg, respectively, while the radial artery pressure averaged 135/71 mm. Hg.

The average statistically significant mean pressure gradients from the antecubital vein, both cavae, and the left atrium to the right atrium were 2, 0.5, and 6 mm. Hg, respectively. The average systolic pressure gradient of 2 mm. Hg across the pulmonary valve was statistically significant.

The pressures during expiration in the right atrium, right ventricle, pulmonary artery, pulmonary artery wedge, and radial artery averaged 1, 2, 3, 3, and 4 mm. Hg, respectively, higher than during the inspiratory phase of the respiratory cycle.

The average maximal differences in systolic pressures recorded at different times in the same individual during cardiac catheterization averaged 2, 4, 3, and 3 mm. Hg in the right atrium, right ventricle, pulmonary artery, and wedge positions, respectively, and ranged from 0 to 12 mm. Hg.

The effect of the administration of 95 per cent oxygen was investigated in 20 of these subjects. There was a fall in heart rate and an increase in stroke volume with no systematic change in cardiac output under these conditions. In addition, a fall in pressure throughout the right heart and pulmonary vascular tree and a rise in systemic arterial pressure were noted.

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SURVIVAL TIME OF THE ERYTHROCYTE IN MYXEDEMA AND HYPERTHYROIDISM

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THE in vivo survival time of erythrocytes in various clinical states has been measured by means of the Ashby technique[1] and by the use of erythrocytes tagged with isotopes, such as chromium^{51}.[2] The normal survival time of erythrocytes as determined by these methods has been found to be 120 ± 15 days. The survival time of the erythrocytes has been shown to be shorter than normal in many clinical states associated with anemia.[3] In some diseases, such as leukemia and lymphosarcoma, the anemia has been found to be associated with an increased basal metabolic rate[4, 5] as well as with decreased survival time of the erythrocytes.[6] It is the purpose of this paper to report the results obtained with the Ashby and chromium^{51} techniques in a study of the survival of erythrocytes in other patients with abnormal metabolic states, namely, myxedema and hyperthyroidism. Approximately one-half of the patients with myxedema have a normocytic or macrocytic anemia which is rarely severe unless there is a concomitant deficiency of iron or vitamin B_{12}.[7] Although anemia is much less common in hyperthyroidism than in myxedema, a mild macrocytic anemia has been reported in as many as one-third of the cases.[8]

MATERIALS AND METHODS

The diagnosis of myxedema in the 5 patients who were studied was based on the clinical manifestations of the disease, supported by a diminished 24 hour uptake of radioactive iodine, a decreased basal metabolic rate, and an elevated serum cholesterol level. The myxedema was not thought to be secondary to hypopituitarism in any instance. The diagnosis of hyperthyroidism was made in the 5 patients on the basis of an elevated 24 hour radioactive iodine uptake and an increased basal metabolic rate, associated with the clinical features of hyperthyroidism. The pertinent data on these patients are shown in Table I. The normal subjects were volunteer male medical students and healthy hospital personnel.

Erythrocytes from 4 patients with myxedema, one of whom was moderately anemic (J. S.), were transfused into normal recipients and the survival time of these cells was measured by the Ashby technique. The same technique was

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used to measure the survival time of erythrocytes from a normal donor that were transfused into one patient with myxedema (V. G.), who was moderately anemic. In studying the survival time of erythrocytes in hyperthyroidism two techniques were employed. Red cells from 3 patients with hyperthyroidism were transfused into normal recipients and the survival times were measured by the Ashby technique. The survival time of the erythrocytes of two other patients with hyperthyroidism was measured by the autotransfusion of chromium[51] tagged erythrocytes.

TABLE I. DATA ON 5 PATIENTS WITH MYXEDEMA AND 5 PATIENTS WITH HYPERTHYROIDISM

CLINICAL DATA					LABORATORY DATA							
PATIENT	AGE	SEX	COL.	DIAGNOSIS	R. A. I. UPTAKE (24 HR.)	HCT. (%)	R. B. C. ($\times 10^6$)	HGB. (GM. %)	RETICS (%)	BILIRUBIN (MG. %)	FECAL UROBILINOGEN, (MG./DAY)	SURVIVAL STUDY
1. E. G.	53	F	W	Myxedema	3.4	49	4.5	15.8	0.4	-	-	Patient to Normal
2. B. P.	62	M	W	Myxedema	5.1	42	4.6	13.0	1.3	0.60	22	Patient to Normal
3. A. P.	46	F	W	Myxedema	6.8	39	4.1	12.6	1.9	0.56	-	Patient to Normal
4. J. S.	57	F	W	Myxedema	7.6	33	3.9	10.2	1.9	0.17	17	Patient to Normal
5. V. G.	58	F	C	Myxedema	10.0	36	3.8	12.0	0.8	0.35	57	Normal to Patient
6. K. D.	44	F	W	Hyperthyroid	75.3	46	5.2	14.9	0.3	0.48	-	Patient to Normal
7. L. P.	50	F	W	Hyperthyroid	61.0	40	4.8	12.5	0.3	0.48	233	Patient to Normal
8. P. T.	50	F	W	Hyperthyroid	60.0	40	4.1	14.0	0.6	0.20	-	Patient to Normal
9. C. J.	34	F	C	Hyperthyroid	67.0	45	5.0	14.7	1.9	0.39	-	Autotransfusion Cr^{51}
10. A. W.	24	F	C	Hyperthyroid	66.0	41	4.9	11.7	1.1	0.73	77	Autotransfusion Cr^{51}

For the routine studies, hematocrits were determined by means of the Wintrobe tube. Erythrocyte counts were made in the usual manner with counting chambers and pipettes that were certified by the United States Bureau of Standards. The hemoglobin values were determined by the use of the Evelyn colorimeter, using 15.6 Gm. of hemoglobin per 100 c.c. of blood as a standard. Reticulocyte counts were made by the indirect method from blood smears stained with brilliant cresyl blue and counter-stained with Wright's stain. Plasma bilirubin levels were measured by the method of Ducci and Watson.[9] Four day stool collections for fecal urobilinogen were considered adequate only if the total weight of the sample was 240 Gm. or more. The method of Schwartz, Sborov, and Watson was used.[10]

In the transfusion studies, the techniques described by Ashby[1] as modified by DeGowin, Sheets, and Hamilton were followed.[11] Certain minor modifications which were suggested by DeGowin and associates to one of the present authors

(C. D.) were made. All of the erythrocyte counts were done on 4 ml. of blood collected in balanced ammonium and potassium oxalate, rather than blood obtained from puncture of the ear lobe. The collection bottles were cleaned carefully and the oxalate was measured and dried. Ashby counts were done on the blood collected in the oxalate, while the hematocrit, hemoglobin, and other determinations were made on additional blood that was placed in a bottle containing heparin. A 1:20 dilution of antiserum was used at all times and only recipients whose "blank" inagglutinable cell counts were below 25,000 per cubic milliliter were used. All donors belonged to group O and all recipients belonged to group A. For all the inagglutinable cell counts on the oxalated blood refrigerated physiologic saline was used as the diluting fluid. The tubes containing the mixture of the antiserum and the red cells suspended in the physiologic saline were centrifuged at 1,800 r.p.m. for one minute and then tapped 20 times with the finger to promote mixing. This procedure was performed 3 times and the top layer of the supernatant fluid was used for counting the inagglutinable cells. In making the total erythrocyte counts 3 erythrocyte pipettes and 3 counting chambers were employed. All 6 sides were counted and the counts were averaged.

The survival time of autogenous red cells was determined by labeling with radioactive chromium as described by Ebaugh, Emerson, and Ross.[2] Fifty milliliters of the patient's blood were suspended in ACD solution in a 75 ml. Fenwal flask and incubated with 75 μc of $Na_2Cr^{51}O_4$ at room temperature for one hour. The plasma was removed by centrifugation and the original volume was reconstituted with sterile physiologic saline prior to injection of the tagged cells with a calibrated syringe. A 1:50 dilution of the donor suspension was made and a 4 ml. aliquot was counted in a flat-bottomed screw-capped vial 15.75 by 50 mm. Since practically all the unbound chromium in the plasma was removed prior to injection, it was unnecessary to correct the first samples for plasma radioactivity. Samples were taken from the recipients at 10- and 20-minute intervals, daily for the first 5 days and weekly after the autotransfusion. Four milliliters of whole blood from the recipients were placed in similar vials and the radioactivity was determined with a well-type scintillation counter after hemolysis had been produced by freezing and thawing of the samples. A chromium standard was counted every second or third sample tested and a correction was made for physical decay and elution in each sample.

RESULTS

Myxedema.—The red cells from 4 patients with myxedema (E. G., B. P., A. P., and J. S.) which were transfused into normal recipients survived a normal period of time in each instance (Fig. 1). One of these patients, (J. S.) had a normocytic anemia of moderate severity. A normal survival time was also found when erythrocytes from a normal donor were transfused into a mildly anemic patient with myxedema (V. G.) while treatment was withheld.

Hyperthyroidism.—Erythrocytes from 3 patients with hyperthyroidism (K. D., L. P., and P. T.) survived normally when transfused into normal recipients (Fig. 2). In 2 patients with marked hyperthyroidism (C. J. and A. W.) who

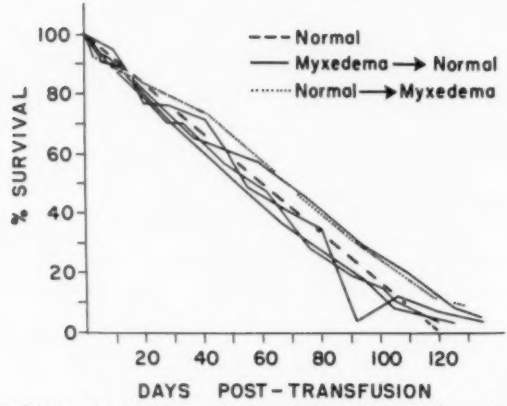

Fig. 1.—Survival time of erythrocytes in 5 patients with myxedema determined by the Ashby technique.

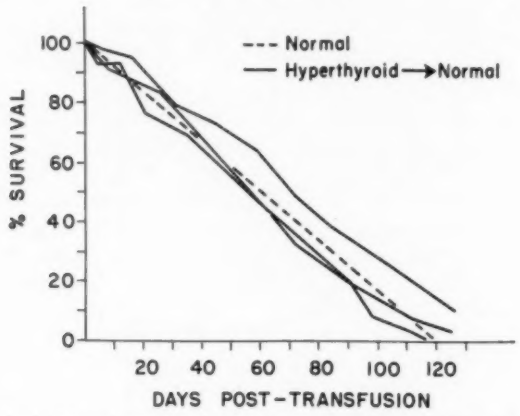

Fig. 2.—Survival time of erythrocytes from 3 patients with hyperthyroidism determined by the Ashby technique.

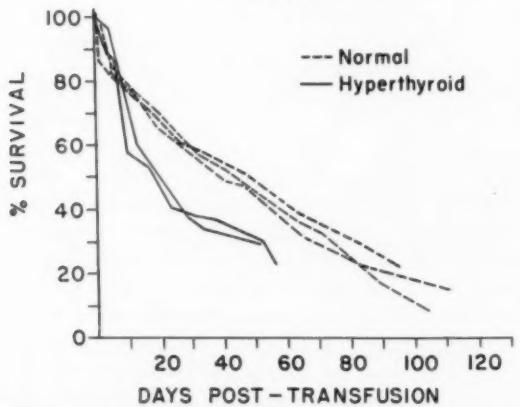

Fig. 3.—Survival time of erythrocytes in 3 normal subjects and 2 patients with hyperthyroidism determined by the chromium^{51} technique.

were studied by the Cr^{51} technique, the 50 per cent survival time of the erythrocytes was found to be approximately 18 days. The survival times of Cr^{51}-labeled autogenous red cells in these 2 patients and 3 normal individuals are shown (Fig. 3). The 50 per cent survival times of the normal patients varied from 39 to 48 days.

DISCUSSION

In the present study, normal erythrocyte survival times were found when erythrocytes from anemic and nonanemic patients with myxedma were transfused into normal recipients. Likewise, erythrocytes from a normal individual survived for a normal time when transfused into a patient with myxedema and anemia while treatment was withheld. Rodnan and Jensen found that the survival time of the erythrocytes in 3 of 8 patients with myxedma was longer than normal when measured in the myxedematous environment by means of the Cr^{51} technique.[12] The results of both studies indicate that hyperhemolysis is not a factor in the anemia of myxedema and support the concept that the anemia of myxedema is due to decreased erythropoiesis.

Erythrocytes from 3 patients with marked hyperthyroidism that were utilized in the present study survived normally when transfused into normal recipients. In 2 patients with hyperthyroidism, shortened erythrocyte survival times were found when the cells were labeled with Cr^{51} and reinjected into the same individuals. Rodnan and Jensen[12] also found a shortened erythrocyte survival time in one of 7 patients with hyperthyroidism studied by means of the Cr^{51} technique. The finding of a diminished survival time of erythrocytes in some patients with hyperthyroidism agrees with Heilmeyer's report of an increased fecal urobilinogen excretion in hyperthyroidism[13] and the evidence of increased erythropoiesis noted in the bone marrow studies of Axelrod and Berman.[14] The absence of anemia in the patients with hyperthyroidism despite the shortened survival times of the erythrocytes is not surprising since the degree of hyperhemolysis is not more than could be compensated by a normal bone marrow which has the capacity to increase erythropiesis sixfold.[15]

The finding of abnormal erythrocyte survival times in some patients with myxedema and hyperthyroidism and not in others was unexpected. The observed differences may be due to differences in the duration or severity of the disorder or to some other factors existing in the individuals who developed dysfunction of the thyroid. Although it is considered very unlikely, it is also possible that these results could be due to technical errors. The finding of a normal erythrocyte survival time in all the myxedema patients by means of the Ashby technique in the present study and Rodnan and Jensen's finding of a prolonged survival time in some of the patients studied with the Cr^{51} technique, suggests that the prolongation of the survival time of the red cells in certain patients is due to alterations in the environment of the erythrocyte rather than some irreversible abnormality inherent in the erythrocyte. The results obtained with the hyperthyroid patients in the present study, that is, normal survival of "hyperthyroid" erythrocytes in normal persons and decreased survival of "hyperthyroid" erythrocytes in patients with hyperthyroidism, also suggest

that the environmental factors are most important. The responsible factors may be the variation in the amounts of trauma associated with the different circulatory states that exist in normal individuals and those with myxedema or hyperthyroidism. Another possibility to be considered is that in the different metabolic environments the erythrocyte undergoes changes in its own metabolic activity that alter its survival time.

SUMMARY AND CONCLUSIONS

1. The erythrocyte survival times in 5 patients with myxedema and 5 patients with hyperthyroidism are reported.

2. Normal survival times were observed when erythrocytes from patients with untreated myxedema were transfused into normal individuals and when erythrocytes from a normal individual were transfused into a patient with untreated myxedema.

3. Erythrocytes from patients with untreated hyperthyroidism survived normally when transfused into normal individuals. Erythrocytes labeled with Cr^{51} and reinfused into 2 patients with hyperthyroidism had a shortened survival time.

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POLIOMYELITIS AND ASEPTIC MENINGITIS

A Two-Year Field and Laboratory Study in Connecticut

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INTRODUCTION

THE diagnosis of poliomyelitis, and more particularly the nonparalytic form of the disease, is frequently made in patients who subsequently show no laboratory evidence of poliomyelitis.[1-10] With the widespread use of poliovaccine, the need for an accurate diagnosis is heightened in order that illness in vaccinated cases be properly evaluated. With the advent of tissue culture techniques, the laboratory is able to function with increasing effectiveness in identifying viral agents associated with aseptic meningitis. Presented here is a laboratory study of reported and suspected cases of poliomyelitis occurring in Connecticut in 1955 and 1956, and certain ancillary investigations.

The study is divided into the following parts: (1) Laboratory data from paralytic cases. (2) Laboratory data from cases of aseptic meningitis including virus isolations in tissue culture and suckling mice, and antibody tests for poliomyelitis, mumps, and certain echo and Coxsackie viruses. (3) Distribution of cases according to age, sex, geography, and season. (4) Clinical and family contact studies (from nonparalytic poliomyelitis, from paralytic poliomyelitis, and from aseptic meningitis due to echo virus type 6, Coxsackie virus types A9 and B3). (5) Serologic surveys of antibodies against poliomyelitis and certain Coxsackie and echo viruses.

MATERIALS AND METHODS

A stool and a blood sample were collected in the acute phase of illness and convalescent blood was collected several weeks later from as many cases of poliomyelitis in Connecticut as possible. From certain cases, cerebrospinal fluid was also tested for virus.

In 1955, a high incidence year, there were 654 cases reported of which 203 were noted as paralytic.[11] Specimens were collected from 58 paralytic cases and 137 nonparalytic cases, hereafter referred to as cases of aseptic meningitis. Most of those from 0 to 14 years of age were included. A few of the paralytic cases and about one-third of the aseptic meningitis cases were merely suspected as being poliomyelitis, and these were not reported to the health department by the practicing physicians.

In 1956, 79 of the 83 (95 per cent) reported cases were studied. In addition, a number of suspected cases of poliomyelitis were included. Clinical records were available on most of the cases, and from some of those showing paralysis, muscle evaluations during convalescence were available.

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A paralytic case is defined in this study as any case so diagnosed by the attending physician unless convalescent muscle evaluation indicated no evidence of residual paralysis. No such confirmatory data were available for the 1955 cases; however, information was obtained from all but 2 paralytic cases reported and studied in 1956.

Cases of aseptic meningitis are defined as those showing no other obvious clinically associated illness that might produce secondary central nervous system manifestations, having no paralytic sequelae of the illness, and demonstrating two or more of the following features: (1) an acute self-limiting febrile illness, (2) clinical meningeal irritation, and (3) cerebrospinal fluid pleocytosis with normal sugar determinations and negative routine bacteriologic culture.

Viruses were isolated and identified in monkey kidney tissue culture by methods already described.[12-14] Poliomyelitis complement-fixing (C.F.)[15] and neutralizing (Nt)[16] antibody determinations were made on all sera. Nt antibody titrations in plastic panels were carried out using fourfold dilutions of sera against 100 TCD_{50} of virus. Nt antibody determinations against the Coxsackie and echo viruses were made using the cytopathic inhibition test or plaque reduction test.[13]

TABLE I. VIRUS ISOLATIONS FROM PARALYTIC POLIOMYELITIS AND ASEPTIC MENINGITIS USING MONKEY KIDNEY TISSUE CULTURE

	1955		1956		TOTAL	
VIRUS	PARALYTIC POLIOMYELITIS	ASEPTIC MENINGITIS	PARALYTIC POLIOMYELITIS	ASEPTIC MENINGITIS	PARALYTIC POLIOMYELITIS	ASEPTIC MENINGITIS
No. studied	58	137	20†	82	78	219
Isolations	45	68	18†	30	63	98
Polio-1	39	27	16†	4	55	31
Polio-2	2	0	0	0	2	0
Polio-3	1	1	2	2	3	3
Echo 1-4	0	0	0	0	0	0
Echo 5	0	1	0	0	0	1
Echo 6	1*	22	0	9	1	31
Echo 7-13	0	0	0	0	0	0
Echo 14	0	2	0	2	0	4
Cox. A9	1*	1	0	6	1	7
Cox. B1	0	0	0	0	0	0
Cox. B2	0	5	0	1	0	6
Cox. B3	0	2	0	4	0	6
Cox. B4	1	7	0	2	1	9
Cox. B5	0	0	0	0	0	0

*Though a poliovirus was not isolated, serologic studies were characteristic of polio infection.

†No stool was collected from one of these cases before death, but a type 1 poliovirus was isolated from another member of the household, who also developed paralysis.

RESULTS

Part 1: Paralytic Cases.—Viral isolations from stool specimens are listed in Table I. Of the 58 cases reported as paralytic in 1955, 42 of 45 viruses isolated were poliovirus (39 type 1, 2 type 2, and 1 type 3). From one case only echo virus type 6 was isolated, but this patient showed serologic evidence of recent infection with poliovirus type 1 as well. From another case only Coxsackie A9 was isolated, but he also showed serologic evidence for recent poliomyelitis. Furthermore, from this patient's brother a type 1 poliovirus was isolated. There was no evidence for recent poliomyelitis in the case excreting the Coxsackie B4 virus; however, only an acute serum was obtained. The records of follow-up muscle evaluation were not obtained on this case nor on the other paralytic cases in 1955.

TABLE II. PARALYTIC POLIOMYELITIS (1956)

CASE NO.	AGE	MONTH OF ONSET	EXTENT OF PARALYSIS	SALK VACC.	STOOL ISOL.	DAYS BETWEEN ONSET AND BLEEDING	C.F. ANTIBODIES			NEUTRALIZING ANTIBODIES		
							IΔ	I	POOLΔ	I	II	III
1.	35	April	Legs*	None	Neg.	5	0	0	0	16	<4	16
						38	0	0	0	16	<4	16
2.	31	July	Legs*	None	Neg.	14	0	0	0	64	1,024	256
						47	0	0	0	64	1,024	256
3.	3	July	Rt. arm and legs	X1†	I	8	12	0	10	256	16	<4
						36	12	8	8	256	16	<4
4.	1	Aug.	Neck‡	X1†	I	2	4	0	8	256	16	<4
						12	18	4	16	256	16	<4
5.	2	Aug.	Rt. arm	None	I	6	0	0	0	1,024	<4	<4
						17	4	4	6	1,024	<4	<4
6.	14	Aug.	Bulbar fatal	None	III	1	0	0	0	<4	<4	64
7.	11	Aug.	Legs	None	I	1	6	32	32	256	<4	<4
						20	32	32	32	256	<4	<4
8.	4	Aug.	Bulbar	X2	I	2	8	8	8	256	256	16
						13	32	32	32	256	256	8
9.	4	Aug.	Legs	X2	I	7	12	4	12	64	16	<4
						36	32	32	32	1,024	1,024	<4
10.	<1	Aug.	Rt. leg	None	I	2	0	0	0	64	<4	<4
						60	8	32	0	256	<4	<4
11.	11	Aug.	Rt. leg	X3 (1954)	I	15	32	32	32	256	16	<4
						34	32	32	32	256	64	<4
12.	13	Sept.	Bulbar and arms	None	I	3	0	0	0	64	<4	<4
						15	16	0	0	1,024	16	8
13.	11	Sept.	Lt. leg	X2	I	7	12	12	12	256	256	1,024
						26	4	8	6	256	256	1,024
14.	23	Sept.	Bulbar fatal	None	I	11	8	0	8	64	1,024	1,024
15.	4	Sept.	Rt. leg	None	III	17	4	6	4	1,024	256	256
16.	18	Sept.	Bulbar	None	I	4	0	0	0	256	<4	<4
						32	4	16	8	256	<4	<4
17.	40	Sept.	Bulbar fatal§[3]	None	‖	5	4	0	4	1,024	<4	<4
18.	10	Sept.	Rt. leg	None	I	4	Ac	Ac	Ac	<64	<64	1,024
						26	0	8	4	1,024	1,024	1,024
19.	25	Sept.	Trunk‡§	X1†	I	5	32	0	0	256	64	8
						93	4	4	0	256	64	256
20.	38	Sept.	Bulbar and lt. arm	None	I	4	0	0	0	256	<4	<4
						23	16	24	6	1,024	<4	<4

*No muscle evaluation or follow-up on paralytic status.
†Vaccinated within one month prior to onset of illness.
‡Reported as nonparalytic, developed paralysis late in acute phase.
§Pregnant in acute phase of illness.
‖No stool collected before death, but Case 16 lived in same house and is the daughter of Case 17.

In 1956, there were 31 reported paralytic cases, all of which were investigated. From the results of the muscle evaluations, 11 were reclassified by us as aseptic meningitis. From 2 of the 20 paralytic cases studied (listed in Table II), there was no available record of muscle testing, and specimens from these 2 cases showed no evidence for recent poliomyelitis. There were 18 cases showing definite weakness on convalescent muscle testing and from 17 of these polioviruses were isolated (15 type 1 and 2 type 3). One of the cases died before a stool could be obtained, but a type 1 poliovirus was isolated from her daughter, also a paralytic case. Two of these 18 cases were reported in the acute phase of illness as nonparalytic. On subsequent examination paralysis was noted.

The two unconfirmed cases were studied early in the season; both were adults and both failed to show any C.F. antibodies. Nt antibodies were present for the polioviruses, but no rise was demonstrable. The 18 confirmed cases of paralytic poliomyelitis were limited to the period from July through September. The age range was 1 to 40 years, with 5 being 18 years or older. Two of these were known to be pregnant at the time of infection. There were 3 deaths; these occurred in patients 14, 23, and 40 years of age, respectively. None of the fatal cases had received poliovaccine.

Concerning cases in vaccinated persons, one paralytic case had received 3 doses of vaccine (in the 1954 field trial), one had received 2 doses in 1955 and two received their 2 doses in 1956. Three had received one dose in 1956, All 3 in the latter group had been vaccinated from the same vaccine lot within one month of the time of onset of illness. Approximately 20,000 doses from this lot had been distributed during the summer of 1956 in Connecticut, these 3 being the only known clinical cases who received this lot. A small portion of the same lot of vaccine was obtained and tested in 12 precortisonized monkeys[17] with negative results.

C.F. antibodies were demonstrable in all 18 confirmed cases with significant elevations noted in 11 of 13 paired sera tested. Nt antibodies for the virus isolated were demonstrable in all 18 cases, with significant elevations noted in 3 of 14 paired sera tested. The failure to demonstrate a significant antibody rise in more cases can be attributed in most instances to late collection of the acute sera.

TABLE III. VIRUS ISOLATIONS USING MONKEY KIDNEY TISSUE CULTURE FROM PARALYTIC POLIOMYELITIS AND ASEPTIC MENINGITIS STUDIED IN 1955 AND 1956 BY AGE AND VACCINATION STATUS

VIRUS ISOLATED	0 to 14 AGE GROUP				OVER 14 AGE GROUP		TOTAL	
	VACCINATED		NONVACCINATED					
	PARALYTIC POLIOMYELITIS	ASEPTIC MENINGITIS	PARALYTIC POLIOMYELITIS	ASEPTIC MENINGITIS	PARALYTIC POLIOMYELITIS	ASEPTIC MENINGITIS	PARALYTIC POLIOMYELITIS	ASEPTIC MENINGITIS
No. studied	13	77	41	72	24*	70	78	219
Isolations	10	35	37	37	16*	26	63	98
Poliovirus	10	6	34	17	16*	11	60	34
Coxsackie virus	0	9	2	8	0	11	2	38
Echo virus	0	20	1	12	0	4	1	36

*No stool was collected from one of these cases before death, but a type 1 poliovirus was isolated from another member of the household who also developed paralysis.

Table III lists the paralytic cases, combining the 2 years' study and separating the 0 to 14 year age group from those over 14 years of age. In the 0 to 14 age group, those having received vaccine were distinguished from those nonvaccinated in the same age group. Insufficient vaccine had been distributed in the older age group to warrant such an analysis.

Aside from the 7 paralytic cases in 1956 who had received vaccine, 6 additional cases that occurred in 1955 were studied. From 3 of these a type 1 poliovirus was isolated (one vaccinated 3 times in the 1954 field trial, one vaccinated twice in 1955 and one vaccinated once in 1955). The remaining 3 cases showed no laboratory evidence for recent poliomyelitis and records of convalescent muscle evaluations were not available.

Approximately 75 per cent (459,000 of 617,000) of the 0 to 14 age group had received 1 or more injections of vaccine prior to July 1, 1956, or the midpoint of this study.[18] With most of the cases in the 0 to 14 age group included in this study, the fact that 34 of 44 confirmed paralytic cases had not received any vaccine* lends strong support for the effectiveness of the vaccine in preventing the paralytic form of the disease. In addition, the majority of the vaccinated population had not received the full 3 doses of vaccine.

There were 24 cases studied in the 15 years and older age group which were reported as showing paralysis. Sixteen of these yielded a virus, all being poliovirus.

Part 2: Aseptic Meningitis Cases.—

Tissue culture isolations: In 1955, 137 cases of aseptic meningitis were studied, all being suspected poliomyelitis and many being reported as nonparalytic poliomyelitis. Stools from these cases yielded 68 (50 per cent) viruses. Of these, 28 (41 per cent) were polioviruses (27 type 1, and 1 type 3), 25 (37 per cent) echo viruses (1 type 5, 22 type 6, and 2 type 14), and 15 (22 per cent) Coxsackie viruses (1 A9, 5 B2, 2 B3, and 7 B4).

There were 82 cases studied in 1956 with 30 (37 per cent) yielding viruses. Of these, 6 (20 per cent) were polioviruses (4 type 1 and 2 type 3), 11 (37 per cent) echo viruses (9 type 6 and 2 type 14), and 13 (43 per cent) Coxsackie viruses (6 A9, 1 B2, 4 B3, and 2 B4). These are listed in Table I. Poliovirus type 1 and echo virus type 6 were the viruses most frequently isolated from this group.

Very few cerebrospinal fluids were submitted for purposes of isolating viruses, but from 2 patients excreting a Coxsackie A9 virus, the same type virus was also isolated from the spinal fluids. The remaining spinal fluids tested, mostly from cases whose stools yielded no detectable virus, were negative.

In Table III, the aseptic meningitis patients are listed according to age and vaccination status. In the vaccinated 0 to 14 age group, 35 of 77 yielded a virus of which only 6 were poliovirus, an incidence of 1.3 per 100,000; of the nonvaccinated cases in this age group, 37 of 72 yielded a virus, of which 17 were polioviruses, an incidence of 10 per 100,000. These rates, together with those given above, suggest that the vaccine was effective against both paralytic and nonparalytic poliomyelitis.

*An incidence of 22/100,000 as opposed to 2/100,000 for the vaccinated 0 to 14 age group. Had all of the cases occurring in this age group been studied, the remaining would have been almost entirely limited to the unvaccinated group; thus any correction factor would merely add more confirmed cases to the unvaccinated group and increase its rate.

In those over 14 years of age, 26 of 70 yielded viruses, of which 11 were polioviruses. There were no significant differences in the distribution of echo and Coxsackie virus isolation in the vaccinated and nonvaccinated groups or the two age groups, with the possible exception that the frequency of echo virus isolation in the adult group was slightly decreased.

TABLE IV. LABORATORY CONFIRMED CASES OF NONPARALYTIC POLIOMYELITIS (1956)

CASE NO.	AGE	MONTH OF ONSET	SALK VACC.	STOOL ISOL.	DAYS BETWEEN ONSET AND BLEEDING	C.F. ANTIBODIES			NEUTRALIZING ANTIBODIES		
						IΔ	I	POOLΔ	I	II	III
1.	12	June	None	I	6	32	0	32	64	<4	16
					18	16	4	32	256	<4	<4
					36	16	16	16	1,024	<4	<4
2.	23	July	None	III	4	0	0	0	<4	<4	64
					13	0	4	6	<4	<4	64
3.	31	Aug.	None	I	7	0	0	0	256	4	<4
					12	4	4	6	256	4	<4
4.	5	Aug.	Twice*	III	2	0	0	0	16	64	256
					16	0	0	0	16	64	1,024
5.	23	Aug.	None	I	14	0	0	0	256	64	1,024
					17	0	0	0	256	64	1,024
6.	13	Sept.	None	I	2	6	0	4	256	16	<4
					22	6	8	4	1,024	16	<4

*Last vaccine inoculation given within one month of onset of illness.

In 1956, there were 51 reported nonparalytic cases of which 48 were studied. An additional 11 were added from the group reported as "paralytic," for no residual paralysis could be found. Six of the 59 were found to be excreting a poliovirus and are listed in Table IV. Two were found to be type 3, one having been vaccinated twice prior to onset of illness. However, the last vaccination was given within a month of onset of illness. The other 4 cases were excreting a type 1 poliovirus. With the exception of one case, age 5, all were over 12 years of age.

Cases 4 and 5 showed no C.F. antibodies using either live type 1 virus or a pool of heated types 1, 2, and 3 viruses. We have noted an occasional poliomyelitis infection not demonstrating detectable C.F. antibodies. On the other hand, the presence of C.F. antibodies may not always represent recent poliomyelitis infection, for immunization with killed poliovirus may also stimulate a C.F. antibody response in some persons.

Nt antibodies were present for the virus excreted in each case, though only Case 1 showed a significant rise in titer, the acute sera in the other cases having been collected too late in the course of infection.

Because a number of the cases studied failed to yield a virus and were also negative serologically for recent poliomyelitis, they were studied further.

Mumps encephalitis: A number of paired sera were analyzed for demonstrable mumps C.F. antibody rise. Of 58 paired sera, the donors of which

failed to yield a viral agent in tissue culture, 3 showed a C.F. response characteristic of recent mumps infection. An additional 25 paired sera, from donors excreting a viral agent, failed to show a significant rise in antibodies for mumps. These results are shown in Table V.

TABLE V. IDENTIFICATION OF POTENTIAL ETIOLOGIC AGENTS FROM CASES WHO FAILED TO YIELD A VIRAL ISOLATION IN MONKEY KIDNEY TISSUE CULTURE

	NO. PAIRED SERA SHOWING RISE IN MUMPS ANTIBODIES	NO. STOOLS YIELDING COXSACKIE VIRUS IN INFANT MICE
Patients yielding no virus in tissue culture	3/58	5 /63
Control patients yielding virus in tissue culture	0/25	5*/27

Numerator = number postive; denominator = number tested.

*All 5 stools were from patients excreting Coxsackie virus (2 type A9, 2 type B3, and 1 type B4). Stools from 5 additional patients, yielding Coxsackie virus in tissue culture (3 type, A9, 1 type B3, and 1 type B4) failed to produce disease in infant mice.

Coxsackie virus isolations in infant mice: Stools found to be negative in tissue culture were inoculated into 1-day-old mice. The Coxsackie A viruses (other than A9) that cannot be isolated in rhesus monkey kidney tissue culture, can often be demonstrated by this means. Of 63 stools negative in tissue culture, 5 produced paralysis in suckling mice. In addition, 27 stools, from which viral agents were isolated in tissue culture, were inoculated into mice also, and 5 of them caused disease, all being from stools yielding Coxsackie virus in tissue culture (2 type A9, 2 type B3, and 1 type B4). However, 5 other stools from this same group of controls, also yielding Coxsackie viruses in tissue culture, failed to produce disease in mice (3 strains were type A9, 1 type B3, and 1 type B4). A higher sensitivity of the tissue culture technique for isolation of these Coxsackie types may well be the explanation.

Clinical features: Clinical records were available on many of the cases of aseptic meningitis. The major features of the symptomatology of the groups according to virus isolations are listed in Table VI.

The most frequent symptoms noted were fever, headache, and stiff neck. Occurring in about half the cases were stiff back, sore throat, back pain, muscle pain, vomiting, and constipation. There was no significant difference in symptomatology between the aseptic meningitis groups, whether they were excreting a poliovirus, Coxsackie group A, Coxsackie group B, echo virus, or were showing no detectable viral agent in the acute stool.

Routine cerebrospinal fluid examinations were made on most patients. In all instances, bacterial cultures were negative as were chemical determinations, with the exception of an occasional slight elevation in protein. The majority of the cerebrospinal fluids showed a predominance of lymphocytes. The only consistent abnormality noted in the fluids was pleocytosis, and these were not significantly different in the groups according to virus isolation. The majority of the cell counts fell in the 25 to 99 W.B.C. per c.mm. group, with a range from 0 to 500 W.B.C. per c.mm.

TABLE VI. RELATIONSHIP OF CLINICAL FEATURES OF ASEPTIC MENINGITIS TO VIRAL ISOLATIONS

	VIRAL ISOLATIONS IN MONKEY KIDNEY TISSUE CULTURE (PER CENT)				
	POLIOVIRUS	COXSACKIE A	COXSACKIE B	ECHO TYPE 6	NEGATIVE
Number studied	26	11	18	29	43
Fever	96	91	89	100	89
Stiff neck	89	82	89	86	75
Headache	77	36	89	83	70
Stiff back	54	55	28	41	44
Sore throat	50	45	33	28	23
Back pain	34	18	28	28	16
Muscle pain	34	18	28	21	27
Vomiting	42	9	39	69	23
Constipation	23	18	5	0	16
Diarrhea	0	0	0	3	0
Cerebrospinal Fluid Pleocytosis					
Number studied	29	6	16	25	39
<10 cells	10	0	19	0	13
10-24 cells	3	0	6	12	10
25-99 cells	45	50	37	52	27
100-300 cells	31	33	13	24	39
>300 cells	10	17	25	12	10
>10 cells	90	100	81	100	87

TABLE VII. GEOGRAPHIC, AGE, AND SEX DISTRIBUTION OF CASES STUDIED IN 1956 ACCORDING TO VIRAL ISOLATIONS

	TOTAL CASES STUDIED		POLIOMYELITIS		COXSACKIE		ECHO	
	NUMBER	ATTACK* RATE	NUMBER	ATTACK* RATE	NUMBER	ATTACK* RATE	NUMBER	ATTACK* RATE
Geographic Distribution								
Fairfield Co.	34	5.8	8	1.4	3	0.5	2	0.3
New Haven Co.	12	1.7	4	0.7	2	0.3	2	0.3
Litchfield Co.	10	9.0	3	2.7	1	0.9	1	0.9
Hartford Co.	35	5.1	4	0.6	6	1.0	6	1.0
Middlesex Co.	1	1.3	1	1.3	0	0.0	0	0.0
Tolland Co.	1	1.8	1	1.8	0	0.0	0	0.0
Windham Co.	3	4.4	2	2.9	0	0.0	0	0.0
New London Co.	6	3.6	1	0.6	1	0.6	0	0.0
Total	102	4.4	24	1.0	13	0.6	11	0.5
Age Distribution								
0-4 Years	18		7		1		2	
5-9 Years	21		1		2		4	
10-14 Years	24		8		4		2	
>14 Years	39		8		6		3	
Total	102		24		13		11	
Sex Distribution								
Male	59		11		6		9	
Female	43		13		7		2	
Total	102		24		13		11	

*Rate per 100,000 population.

Part 3: Distribution of the Cases in 1956.—Because nearly all cases diagnosed or suspected as poliomyelitis in 1956 were studied in our laboratory, the data could be used for epidemiologic analysis.

Geographic: As shown in Table VII, the 102 cases studied were scattered throughout the state with the 10 from Litchfield County (representing an attack rate of 9 per 100,000) being the highest incidence and the case from Middlesex County (representing 1.3 per 100,000) being the lowest. Of the 24 cases who showed confirmatory laboratory evidence for poliomyelitis, one or more occurred in each of the counties, with a similar attack rate for each. With this being a year of low incidence, these differences are not significant. The attack rate for the state was 1 per 100,000.

The distributions of Coxsackie and echo virus isolations were also uniform. No nonpoliovirus was isolated from the less populated counties of Windham, Tolland, and Middlesex. The over-all incidence of each of these viruses was half that of poliovirus in the group studied.

Age distribution: Most of the cases studied fell into the young age group; however, 39 of 102 were over 14 years of age. The younger cases generally yielded a slightly higher percentage of viral isolations.

Poliovirus was evenly distributed among the age groups with the notable exception of those in the 5 to 9 age group, where only 1 case occurred. More vaccine had been distributed in this age group and this probably explains the low incidence. It is remarkable that in previous years this age group had the highest incidence of poliomyelitis.

From the limited data, there seems to be a tendency for Coxsackie virus to be isolated from the older age group, whereas the echo viruses were evenly distributed.

Sex distribution: Although slightly more males were reported and studied, a somewhat higher number of poliomyelitis and Coxsackie viruses were isolated from the female population. These differences are not significant. However, as regards the echo viruses, where 9 of 11 isolates came from males, there may be a real difference.

TABLE VIII. SEASONAL DISTRIBUTION OF CASES STUDIED AND REPORTED BY MONTH OF ONSET OF ILLNESS AND THEIR RELATION TO VIRUS ISOLATION

MONTH	TOTAL CASES STUDIED	REPORTED CASES STUDIED	LABORATORY CONFIRMED POLIOMYELITIS	NONPOLIOVIRUS ISOLATED FROM REPORTED CASES	TOTAL NON-POLIOVIRUS ISOLATED
Jan.	0	0	–	–	–
Feb.	2	0	–	–	0
Mar.	1	0	–	–	0
Apr.	9	4	0	1	3
May	4	4	0	0	0
June	4	3	1	0	0
July	12	11	2	0	1
Aug.	38	29	12	7	11
Sept.	24	21	9	4	6
Oct.	7	6	0	2	3
Nov.	1	1	0	0	0
Dec.	0	0	–	–	–
Total	102	79	24	14	24

Seasonal distribution: As shown in Table VIII, no case came to our attention until February; from then until November, cases had their onsets in

each of the 10 intervening months, but with 62 of 102 occurring in August and September. Seventy-nine were reported to the Department of Health, the months of onset falling between April and November, with 50 of 79 occurring in August and September. Poliovirus was isolated from 24 of the 79 cases reported, with their monthly distribution being from June through September. There were 14 Coxsackie and echo virus isolations from the reported cases, and an additional 10 nonpolioviruses isolated from suspected but not reported cases. The distribution of their isolations ranged from March through October with only Coxsackie A9 isolations being made early in the season. Thus, only 79 of 102 cases which came to our attention were reported. There were 24 cases confirmed as poliomyelitis, with an additional 24 cases excreting a nonpoliovirus.

TABLE IX. FAMILY STUDY OF NONPARALYTIC POLIOMYELITIS

	AGE (YR.)	SALK VACC.	DATE OF ONSET	INTERVAL BETWEEN ONSET AND COLLECTION (WK.)	TITER OF TYPE 1 POLIO-VIRUS IN STOOL*	POLIOMYELITIS NEUTRALIZING ANTIBODIES			POLIO-MYELITIS C.F. ANTIBODIES	
						TYPE 1	TYPE 2	TYPE 3	TYPE 1Δ	TYPE 1
Clinical case	12	None	6/14/56	1	10^4	64	<4	16	32	<4
				2	10^2	256	<4	<4	16	4
				3	10^3					
				4	10^2					
				5	10^3	1,024	<4	<4	16	16
Contact	6	5/29/56	None	1	10^4	64	8	<4	16	<4
		6/19/56		2	10^2					
				3	10^2					
				4	Neg.					
				5	Neg.	64	8	<4	8	8
Contact	8	3/19/55	None	1	10^3	1,024	1,024	256	Ac†	Ac
		8/ 2/55		2	10^1					
				3	Neg.					
				4	Neg.					
				5	Neg.	256	1,024	16	4	8
Contact	10	5/ 5/54	None	1	Neg.	64	16	<4	Ac	Ac
		5/11/54		2	Neg.					
		6/ 8/54		5	Neg.	64	16	<4	8	4
Contact	16	None	None	1		64	<4	<4	8	<4
				3	10^1					
				5	10^1	256	<4	<4	6	6
Contact	45	None	None	1	Neg.	64	<4	16	<4	<4
				5		64	<4	16	<4	<4
Contact	50	None	None	1	Neg.	16	16	16	<4	<4
				5	Neg.	16	16	16	<4	<4

*TCD_{50} of virus per gram of stool.
†Ac = Anticomplementary.

Part 4. Clinical and Family Studies.—Household contacts of some of the cases were investigated. Representative examples of nonparalytic and paralytic poliomyelitis, and aseptic meningitis caused by echo 6, Coxsackie A9, and Coxsackie B3 are presented here.

Nonparalytic poliomyelitis: J. K. (Table IX), a 12-year-old boy complained of abdominal pain the day after a classroom trip; he vomited once, and was noted to have a temperature of 101° F., followed the next day by a moderate headache. The patient was seen by his physician who noted a temperature of 101.6° F., moderate nuchal rigidity with pain referred to a mid-thoracic spine. Other neurologic signs being negative, questioning revealed that the patient had sustained a blow to the area 4 days previously. On admission to the hospital, x-ray of the thoracic spine was negative but lumbar puncture revealed 50 W.B.C. (30 being lymphocytes) per cubic millimeter with a protein level of 59 mg. per cent. The sugar was normal, and there was no bacterial growth. One week after onset of illness, the patient was discharged asymptomatic and without sequelae. The patient had not received any polio vaccine.

Stools were collected at weekly intervals for 5 weeks and a type 1 poliovirus was isolated from each specimen. The first stool, collected within the first week of onset of illness, contained 10,000 virus particles (TCD_{50}) per gram of stool and during the 4 subsequent weeks the virus concentration ranged from 100 to 1,000 per gram. C.F. antibody determinations on sera collected the first, second, and fifth week after onset showed a fall in titer using heat-killed antigen, but a significant rise using live antigen. The detectable antibody response is slower by the latter method, whereas using the killed antigen, the acute serum was collected too late in the course of infection to be of diagnostic value.[19] Nt antibody determinations for type 1 poliovirus showed a moderately high titer in the first serum, which rose progressively in subsequent sera. This too is a response often initiated before acute blood can be collected. No detectable Nt antibodies were noted to type 2 poliovirus, and only a transitory titer was noted to type 3.

Six contacts were studied, 2 of them adults. A 6-year-old contact had been given Salk vaccine twice during the previous month. This child excreted a type 1 poliovirus for the 3 weeks following onset of illness of the index case. The C.F. response was similar to that of the clinical case; however, Nt antibodies showed no change, maximum response for the period studied having been reached prior to collecting the first serum.

Another contact, age 8, had been vaccinated against poliomyelitis twice in 1955. This child excreted virus for 2 weeks. The first serum was anticomplementary, but the second serum showed C.F. antibodies, although at a low titer. Not only did Nt antibody determinations fail to show a rise, but a slight fall was noted to types 1 and 3.

A third contact, age 10, had been included in the 1954 field trial and although no virus was isolated C.F. antibodies were noted in the late phase serum. Nt antibodies remained unchanged.

A fourth contact, age 16, had not received any poliovaccine. He excreted the virus over the period of study, showed a characteristic C.F. response, and demonstrated a slight elevation in type 1 Nt antibodies. No detectable Nt antibodies were noted to types 2 and 3.

Neither parent of these children received vaccine and neither excreted virus nor showed any detectable C.F. antibodies. Although their Nt antibodies were of low titer, apparently having been derived from previous inapparent infection, they were sufficiently protected from reinfection.

None of the contacts showed any clinical evidence of poliomyelitis during the period of study.

Paralytic poliomyelitis: C. G. (Table X), a 4-year-old boy, was admitted to the hospital with a one-day history of fever and headache, nasal voice, right facial weakness, dysphagia, vomiting, and a fever of 103° F. Cerebrospinal fluid on admission revealed 50 W.B.C. per cubic millimeter, all lymphocytes, and a protein of 48 mg. per cent. Four weeks after onset, only minimal facial weakness persisted. The patient had received 2 doses of Salk vaccine about 5 months prior to onset of illness.

A rectal swab collected on admission, and stools collected on the second and fourth week after onset of illness yielded a type 1 poliovirus. C.F. antibody rise from 8 to 32 was noted from the first to second week of illness using both killed and viable antigen; however, the Nt antibody determinations remained the same, maximum response for the period having

been reached before the first serum was collected. The effect the vaccine might have had on antibody response can only be speculated upon, since serum prior to illness was not collected.

Two siblings, ages 6 and 9, and the mother, had noted "summer grippe" (mild fever and headache lasting 24 hours) during the week preceding the onset of illness in the index case. Stools collected from these contacts during the patient's second week of illness yielded poliovirus. A stool tested from the father, who remained well, was negative. The siblings had received Salk vaccine twice at the same time as the patient, but neither parent had been vaccinated.

Repeat stools collected from the contacts two weeks later were negative. However, the patient who was convalescing satisfactorily at home was still excreting poliovirus at a high concentration.

TABLE X. FAMILY STUDY OF PARALYTIC POLIOMYELITIS

	AGE (YR.)	SALK VACC.	DATE OF ONSET	INTERVAL BETWEEN ONSET AND COLLECTION (WK.)	TITER OF TYPE 1 POLIO-VIRUS IN STOOL*	POLIOMYELITIS NEUTRALIZING ANTIBODIES			POLIO-MYELITIS C.F. ANTIBODIES	
						TYPE 1	TYPE 2	TYPE 3	TYPE 1Δ	TYPE 1
Clinical case	4	2/56	8/56	1	Positive†	256	256	16	8	8
		3/56		2	Positive‡	256	256	16	32	32
				4	10^3					
Contact	6	2/56	None§	2	10^3	256	1,024	<4	32	16
		3/56		4	Neg.					
Contact	12	2/56	None§	2	10^3	256	256	64	32	8
		3/56		4	Neg.					
Contact	40	None	None§	2	10^2	1,024	1,024	256	<4	<4
				4	Neg.					
Contact	44	None	None	2	Neg.	256	16	<4	<4	<4

*TCD_{50} of virus per gram of stool.
†Rectal swab specimen; no titer available.
‡Not titered.
§Minor morbidity symptoms noted about 1 week prior to onset of clinical case. See text.

TABLE XI. FAMILY STUDY OF ASEPTIC MENINGITIS ASSOCIATED WITH ECHO VIRUS TYPE 6 EXCRETION

	AGE (YR.)	INTERVAL BETWEEN ONSET AND COLLEC-TION (WK.)	STOOL ISOL.	NEUTRALIZING ANTIBODIES				POLIOMYELITIS C.F. ANTIBODIES					
				ECHO	POLIOMYELITIS			TITER			AVIDITY		
				TYPE 6	TYPE 1	TYPE 2	TYPE 3	TYPE 1	TYPE 2	TYPE 3	TYPE 1	TYPE 2	TYPE 3
Clinical case*	10	1	Echo-6	10	256	256	256	8	0	0	14	0	0
		2		50	256	256	256	8	0	8	9	0	11
		16		250	256	256	256	16	4	8	16	2	13
Clinical case	13	1	Echo-6	10	64	<4	16	4	0	0	2	0	0
		2		250	256	<4	16	0	0	0	0	0	0
		16		250	256	<4	16	0	0	0	0	0	0
Clinical case	8	1						0	0	0	0	0	0
		2		50	1,024	256	256	0	0	0	0	0	0
		16		250	1,024	256	1,024	0	0	0	0	0	0
Contact	40	16		10	256	1,024	256	0	0	0	0	0	0
Contact	45	16		10	256	256	1,024	0	0	0	0	0	0

*Case had been given Salk vaccine prior to onset of illness.

Aseptic meningitis associated with echo virus type 6: R. B. (Table XI), a 10-year-old boy, developed fever of 104° F. and a sore throat, followed the next day by headache, vomiting, and a stiff neck. On admission to the hospital, cerebrospinal fluid revealed 10 W.B.C./c.mm. with protein at 34 mg. per cent. This patient had received one dose of Salk vaccine 2 months prior to illness.

W. B., a 13-year-old sibling of R. B., had fever of 102° F., stiff neck, headache, and vomiting at the same time. There were 322 W.B.C./c.mm. in his cerebrospinal fluid. F. H., an 8-year-old adopted sibling, was also admitted to the hospital with chest pain, nonproductive cough, vomiting, stiff neck, headache, and fever of 102° F. His cerebrospinal fluid revealed 150 W.B.C./c.mm. The predominant cells were lymphocytes in all three instances.

Symptoms of all three patients spontaneously resolved and by 2 weeks after onset of illness recovery was complete. Parents of these cases remained well throughout the period.

Stools were collected from W. B. and R. B. and bloods were collected from all 3 cases, in the acute phase. Late convalescent bloods were collected from the entire household. Echo-6 virus was isolated from both stool specimens. Nt antibodies to echo-6 showed a rise in titer in all 3 cases. There was no significant change in Nt antibody titer to the polioviruses. Poliomyelitis C.F. antibodies were noted in the case of R. B., but with a history of having received vaccine, negative stool for poliovirus and no evidence for poliomyelitis in other members of the household, it is unlikely that he had a dual polio-echo infection.

Aseptic meningitis associated with Coxsackie virus type A9: R. M. (Table XII), a 24-year-old man, was admitted to the hospital with a one-day history of headache, sore throat, fever, and stiff neck. Cerebrospinal fluid on admission revealed 256 W.B.C./c.mm. (117 lymphocytes) and protein at 60 mg. per cent. The patient was treated symptomatically and discharged within a week asymptomatic and without sequelae. Poliovaccine had not been given.

TABLE XII. FAMILY STUDY OF ASEPTIC MENINGITIS ASSOCIATED WITH COXSACKIE VIRUS TYPE A9 EXCRETION

	AGE (YR.)	INTERVAL BETWEEN ONSET AND COLLECTION (DAYS)	STOOL ISOL.	NEUTRALIZING ANTIBODIES				POLIOMYELITIS C.F. ANTIBODIES
				COXSACKIE	POLIOMYELITIS			
				TYPE A9	TYPE 1	TYPE 2	TYPE 3	
Clinical case	24	2	Cox. A9	50	<4	256	<4	Neg.
		4	Cox. A9					
		6	Neg.	250	<4	1,024	<4	Neg.
Contact	53	6	Neg.		16	64	64	Neg.
Contact	54	6	Neg.		256	16	64	Neg.
Contact	77	6	Neg.					
Contact	23	6	Neg.					
Contact	<1	6	Cox. A9					
Contact	1	6	Neg.					

Stools collected 2 and 4 days after onset of illness yielded a Coxsackie A9 virus, but a stool collected on the sixth day after onset was negative. Stools were collected from 6 members of the household on the sixth day after onset. The patient's parents and grandparents, who remained well, were negative. The patient's wife, then convalescing from an episode of fever and headache, was also negative. One of the patient's children, age 9 months, although well, was excreting a Coxsackie A9 virus. The other child, age 20 months and also well, was negative.

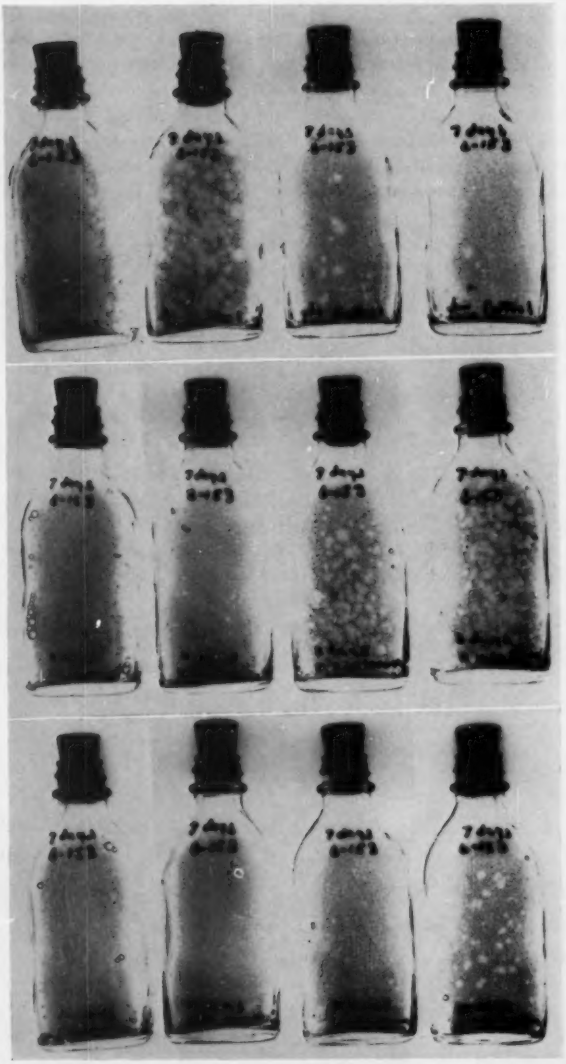

Fig. 1.—Plaque reduction test in bottles. *A*, Virus titration. Inoculum was the third tissue culture passage of the virus isolated from the patient, typed as Coxsackie B3 virus. See Table XIV for details of plaque counts and parallel test in tubes on the same day as bottles pictured here. Virus dilutions (from left to right): 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. *B*, Serum titration in bottles. Test dose 0.1 c.c. 10^{-2} virus. Acute serum—taken 2 days after onset. Serum dilutions (from left to right): 1:10, 1:50, 1:250, and 1:1,250. *C*. Same as Fig. 1,*B* but using convalescent serum taken 12 days after onset. *D*. Same as Fig. 1,*B* but using control serum, prepared in monkeys hyperimmunized with Nancy strain Coxsackie virus B3. Serum dilutions (left to right): 1:100, 1:500, 1:2,500, 1:12,500.

Poliomyelitis Nt antibodies in the patient's sera collected on the first and sixth day after onset were limited to type 2, which titer did not change. No C.F. antibodies were demonstrable in either serum. Nt antibodies for Coxsackie A9 demonstrated a titer of 50 in the acute serum which rose to 250 in the second blood.

Aseptic meningitis associated with Coxsackie type B3: J. A. (Tables XIII and XIV and Fig. 1), a 10-year-old boy, was admitted to the hospital with a one-day history of headache, fever, dizziness, and stiff neck. On admission, slight tenderness was noted over the hamstring muscles. Cerebrospinal fluid revealed 32 W.B.C./c.mm. (14 lymphocytes); repeated on the sixth day, 100 W.B.C./c.mm. (all lymphocytes) were noted. The patient was treated symptomatically and recovery was complete. Two doses of poliovaccine had been administered in 1955, and a booster was given 7 weeks prior to onset of illness.

Fig. 1D. *(For legend see opposite page.)*

TABLE XIII. ASEPTIC MENINGITIS ASSOCIATED WITH COXSACKIE VIRUS TYPE B3 EXCRETION

	AGE (YR.)	INTERVAL BETWEEN ONSET AND COLLECTION (DAYS)	STOOL ISOL.	NEUTRALIZING ANTIBODIES					POLIOMYELITIS C.F. ANTIBODIES
				COXSACKIE B3		POLIOMYELITIS			
				C.P.I.*	PLAQUE COUNT†	TYPE 1	TYPE 2	TYPE 3	
Clinical case	10	2	Cox. B3	<10	50	1,024	1,024	1,024	Neg.
		12		50	> 250	1,024	1,024	1,024	Neg.

*Cytopathic inhibition neutralizing antibody determination by conventional method using monkey kidney tissue culture in test tubes with liquid media, 100 TCD_{50} of virus and inactivated sera at dilutions of 1:10, 1:50, 1:250, and 1:1,250.

†Neutralizing antibody determinations using plaque method, but otherwise the same (Note Fig. 1 and Table XIV). However, 100 TCD_{50} in the conventional method would be found to represent over 1,000 TCD_{50} of virus in the plaque method; thus when 100 TCD_{50} is used in bottles, the virus challenge is actually less, and the measurable titer is greater.

A stool collected 2 days after onset yielded a Coxsackie B3 virus. Nt antibodies for Coxsackie B3 showed a greater than fivefold rise on paired sera collected the second and twelfth days. Nt antibodies for poliomyelitis showed high titers in both sera to all 3 types. C.F. antibodies for poliomyelitis were low and could be attributed to the vaccine given a few weeks before.

Nt antibody determinations were also made using the plaque technique.[13, 14] Being a more sensitive test, allowing smaller virus challenge, and, more significantly, allowing neutralization to be read even in the presence of some nonneutralized virus, antibody titers

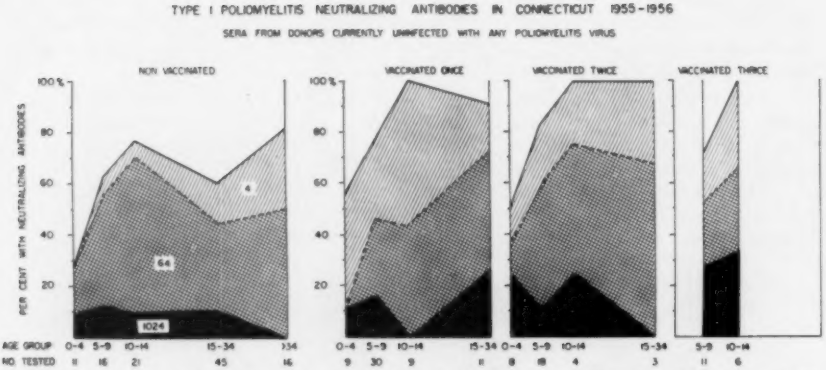

Fig. 2.—Type 1 poliomyelitis neutralizing antibodies in Connecticut 1955-1956. Sera from donors currently uninfected with any poliomyelitis virus. Solid black indicates persons with Nt titer of 1,024 or greater; shading with double lines, titer of 64 or greater; shading with single lines, titer of 4 or greater.

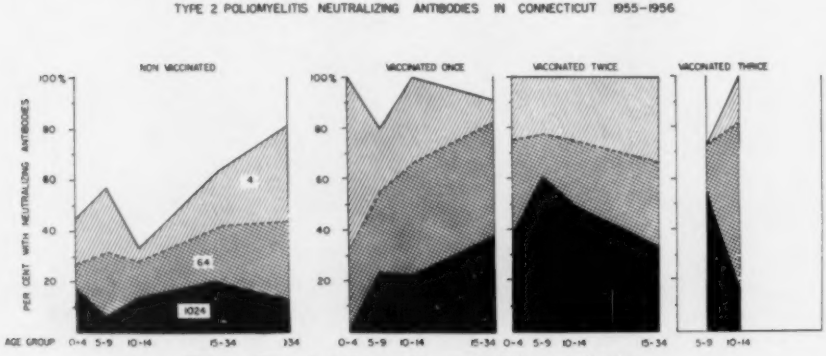

Fig. 3.—Type 2 poliomyelitis neutralizing antibodies.

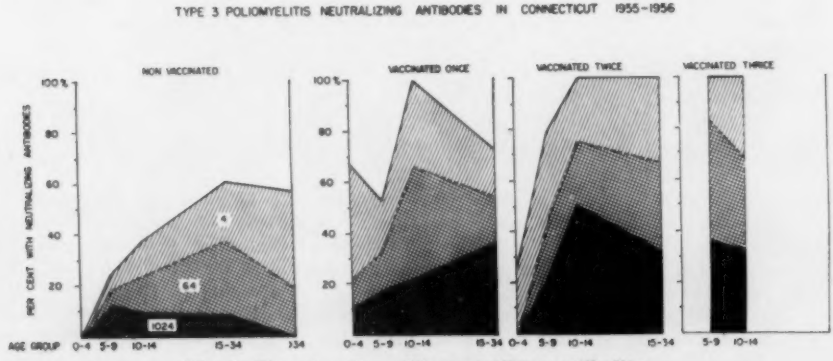

Fig. 4.—Type 3 poliomyelitis neutralizing antibodies.

were found to be higher: 50 in the acute serum and over 250 in the convalescent serum. A control hyperimmune monkey serum tested at the same time was shown to have a titer of over 2,500.

Part 5. Serologic Surveys.—Sera submitted to the laboratory for various purposes were routinely run for Nt antibody titers against poliomyelitis. These sera represented people of all age groups and vaccination status. An attempt was made to demonstrate differences in these groups. Many of the sera were also tested for Nt antibodies against other antigens, including Coxsackie A9, echo-6, and some viruses not commonly found in Connecticut. A few of the sera were also tested for the presence of mumps C.F. antibodies. This has enabled us to obtain a measure of the prevalence of a variety of viral agents. Antibody determinations were not included in the tabulation if the donor showed evidence for recent infection with the virus tested or, in the case of the poliomyelitis survey, with any poliovirus.

TABLE XIV. PROTOCOL TEST USING COXSACKIE VIRUS TYPE B3* IN TUBES AND BOTTLES IN PARALLEL: READING ON SEVENTH DAY AFTER INOCULATION

VIRUS TITRATION		SERUM TITRATION					
		ACUTE SERUM		CONVALESCENT SERUM		CONTROL ANTISERUM	
Cytopathic Inhibition Test in Tube							
10^{-1}	+++	1:10	+++	1:10	000	1:100	000
10^{-2}	+++	1:50	+++	1:50	00+	1:500	+++
10^{-3}	++0	1:250	+++	1:250	+++	1:2,500	+++
10^{-4}	000	1:1,250	+++	1:1,250	+++	1:12,500	+++
Plaque Reduction Test in Bottles (see Fig. 1.)							
10^{-2}	Confluent	1:10	0	1:10	0	1:100	0
10^{-2}	100+ plaques	1:50	17	1:50	0	1:500	0
10^{-4}	14 plaques	1:250	100	1:250	8	1:2,500	20
10^{-5}	3 plaques	1:1,250	100+	1:1,250	38	1:12,500	100+

*Strain 6-153, from patient's stool; third passage.

For the serum titration in tube cultures, the test dose was 0.1 c.c. of 10^{-1} virus; for the plaque reduction test in bottles, the test dose was 0.1 c.c. of 10^{-2} virus.

Poliomyelitis type 1 Nt antibody survey: Fig. 2 illustrates the titer of antibodies for type 1 poliovirus in the various groups according to age and vaccination status. The nonvaccinated group serves as a control to demonstrate the expected pattern without vaccination stimulation. Less than 40 per cent in the group 0 to 4 years of age demonstrated any antibodies, with a progressive rise to about 80 per cent in the group over 34 years of age. Those demonstrating high titers also rose in number up to the age group of 10 to 14 years; then a fall was noted, which probably reflects the decline in antibody level years after exposure to virus.

Although the numbers of sera tested were small, it is apparent that there is an antibody-stimulating factor offered by both age (natural exposure) and vaccine, the latter not being highly efficient quantitatively or qualitatively. There were instances in which after multiple vaccinations, the titer of antibodies was either low or undetectable.

Poliomyelitis type 2 Nt antibody survey: Fig. 3 illustrates the same pattern as found above, except that there appear to be less antibodies among the

TABLE XV. NEUTRALIZING ANTIBODY SURVEY FOR SOME VIRUSES ASSOCIATED WITH ASEPTIC MENINGITIS SYNDROME IN CONNECTICUT 1955-1956*

	POLIOVIRUS†			COXSACKIE VIRUS					ECHO VIRUS				MUMPS‡
	TYPE 1	TYPE 2	TYPE 3	TYPE A9	TYPE B1	TYPE B2	TYPE B3	TYPE B4	TYPE 2	TYPE 5	TYPE 6	TYPE 9	C.F. TITER OF 1:4 OR MORE
Group Under 15 Years of Age													
Neutralizing antibodies of a titer of 1:10 or higher for 100 TCD_{50} of virus	28	17	11	30	17	49	32	34	28	9	22	14	40
Number of donors tested	48	48	48	152	148	148	139	125	151	151	130	115	63
Per cent with neutralizing antibodies	58	35	23	20	11	33	23	27	19	6	17	9	64
Group Over 14 Years of Age													
Neutralizing antibodies of a titer of 1:10 or higher for 100 TCD_{50} of virus	38	34	33	35	4	24	24	35	24	10	16	10	31
Number of sera tested	61	61	61	72	63	64	60	57	66	61	71	52	41
Per cent with neutralizing antibodies	61	56	54	49	6	38	40	61	36	16	23	19	76

*Neutralizing antibodies from donors whose stool was negative or who were excreting a virus other than that being tested.

†Only nonvaccinated donors tested.

‡Mumps C.F. determination carried out by the Connecticut State Health Department Laboratory using the viral antigen. Those donors who demonstrated a fourfold or greater rise in C.F. antibodies were not included in this table.

unvaccinated group, and more among those vaccinated. This confirms a number of observations as to the greater potency of type 2 antigen in the vaccine.

Poliomyelitis type 3 Nt antibody survey: Fig. 4, illustrating the same pattern, shows even more vividly the lack of antibodies in the unvaccinated population.

Selected Coxsackie and echo viruses were used to screen the sera at a 1:10 dilution for the presence of Nt antibodies. Sera showing titers of 1:10 or greater were considered as having previous infection with the agent. The sera donors were grouped into two age categories, those 0 to 14 years of age and persons over 14 years of age. These data, plus comparable poliomyelitis antibody titers from nonvaccinated donors are listed in Table XV.

Fifty-eight per cent (28 of 48) of the unvaccinated children demonstrated antibodies for poliovirus type 1, while 35 per cent (17 of 48) and 23 per cent (11 of 48) demonstrated antibodies to types 2 and 3, respectively. Even though a higher percentage of the adults was positive, 18 per cent (11 of 61) of the adults tested failed to demonstrate *any* poliomyelitis antibodies.

The prevalence of Coxsackie A9 antibodies was essentially the same as for the polioviruses with 20 per cent of the children and 49 per cent of the adults being positive. For the Coxsackie B viruses, using essentially the same sera, 11 per cent of those under 15 years had B1 antibodies, 33 per cent B2, 23 per cent B3, and 27 per cent B4, while among the adults, 6 per cent had B1 antibodies, 38 per cent B2, 40 per cent B3, and 61 per cent B4.

Antibodies against 4 echo viruses were tested in a similar manner. Among those under 15 years of age, 19 per cent showed echo-2 antibodies, 6 per cent echo-5, 17 per cent echo-6, and 9 per cent echo-9, while among the adults the corresponding figures were 36, 16, 23, and 19 per cent, respectively.

Mumps C.F. antibody determinations were carried out, using the viral antigen, on 104 sera donors. Among the children, 64 per cent showed antibodies while 76 per cent of the adults demonstrated antibodies.

DISCUSSION

Found by other observers and reflected in our data is the high degree of laboratory confirmation of *paralytic* poliomyelitis and the low frequency of poliovirus infection among cases of aseptic meningitis, all too often reported as nonparalytic poliomyelitis. Thus any evaluation of the incidence of poliomyelitis must consider seriously to what extent nonpoliomyelitis infections are included. The poliovaccine has no influence on the occurrence of aseptic meningitis due to these echo and Coxsackie viruses.

The frequency of isolation of Coxsackie and echo viruses from aseptic meningitis cases, both from stools and occasionally from cerebrospinal fluids, and in endemic and epidemic occurrences, has led to the belief that these viruses may represent true etiologic agents of this syndrome. The age, sex, and seasonal

distribution, as well as the clinical course of the illness, simulates that of confirmed nonparalytic poliomyelitis. Families into which these agents were introduced, regardless of viral type, demonstrated the apparent ease with which these agents spread and the high frequency of asymptomatic infection, again reflecting the similarity of these enteric viruses.

Among those tested in Connecticut, antibodies for the nonpolioviruses were found less prevalent than for the polioviruses. This may be a true reflection of their lesser prevalence, or the weaker antigenicity of the Coxsackie and echo viruses in stimulating a detectable titer using the techniques followed in this study, or it may indicate that the antibody response is relatively transitory. The fact that most cases occurred in children, although the adults showed a higher prevalence of antibodies, tends to lessen the likelihood of the latter possibility. However, it must be appreciated that antibody levels found might vary with the technique used and also with the antigen inducing the infection. As for poliomyelitis, antibodies of lower titers were noted among vaccinated children and unvaccinated adults as compared with recently infected children.

SUMMARY

Data collected over a 2-year period in Connecticut from cases of poliomyelitis and aseptic meningitis are presented. Poliomyelitis cases whose paralytic status was established by muscle evaluation during convalescence were readily confirmed by laboratory tests for virus and antibody. There were instances in which poliomyelitis occurred among the Salk-vaccinated population, but the attack rate for all forms of laboratory confirmed clinical poliomyelitis was much less in the vaccinated group.

From cases of aseptic meningitis, all presenting essentially the same clinical features, a variety of viral agents were isolated. Poliovirus type 1 and echo virus type 6 were the most common agents encountered among the isolations in monkey kidney tissue culture. However, a variety of agents were encountered, depending on the tests employed. Coxsackie viruses that showed no cytopathogenic changes in tissue culture, could be isolated by using suckling mice, yet other stools containing Coxsackie virus detectable in tissue culture failed to produce paralysis in mice in some instances. Coxsackie A9 was isolated in two instances from the cerebrospinal fluid of patients who were also excreting the virus in their stools. From a few of the cases in which no viral agent could be isolated, paired sera showed laboratory evidence for recent mumps infection.

Contact studies revealed that the attack rate for the echo-6 and Coxsackie A9 and B3 viruses in a family was high, similar to that of poliovirus.

Serologic surveys were carried out for the polioviruses as well as a number of the other enteric viruses (echo types 2, 5, 6, and 9, and Coxsackie B1, B2, B3, B4, and A9) demonstrating a similarity in prevalence for many but not all of these agents.

In confirmation of many other observations, poliomyelitis vaccine increased the polio antibody prevalence and decreased the incidence of clinical

poliomyelitis in the vaccinated group. In contrast, the vaccine had no effect on prevalence of aseptic meningitis due to Coxsackie or echo viruses.

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ARTERIOVENOUS SHUNTING IN PALMAR ERYTHEMA; THE EFFECT UPON BLOOD AMMONIA DETERMINATIONS

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THE association of palmar erythema with arteriovenous shunting of blood in the hand has been demonstrated by Silverstein[1] in patients with hepatic cirrhosis. In his studies, exclusion of the erythematous palm from the circulation resulted in a marked fall in the oxygen content of antecubital vein blood in contrast to a slight decrease observed in patients without palmar erythema. This observation may be of considerable significance in studies of tissue metabolism as measured by arteriovenous differences when the antecubital vein is used. As a specific example, in studies of ammonia metabolism in hepatic disease the possibility exists that with the use of antecubital venous blood the presence of significant arteriovenous shunts in the erythematous palm may lead to falsely high ammonia levels.

Bessman and Bradley[2] have noted that arterial blood ammonia concentrations are significantly greater than venous concentrations and accordingly have postulated that peripheral utilization of ammonia occurs. In venous catheterization studies Faloon and associates[3] have demonstrated a rise in blood ammonia concentration across the lungs, thereby suggesting the possibility that increasing the oxygen concentration of blood may yield an increase in the apparent blood ammonia value. This effect of oxygen upon the blood ammonia determination has also been noted in in vitro studies.[4, 5] Regardless of whether peripheral utilization, oxygen effect, or a combination of these represents the true interpretation of the findings, the shunting of arterial blood into veins in patients with palmar erythema may introduce an error in studies in which blood ammonia is measured in the antecubital vein.

In an attempt to determine the extent to which palmar erythema does affect venous blood ammonia values studies of blood ammonia and oxygen in patients with and without palmar erythema have been performed.

MATERIAL AND METHODS

A total of 19 studies were performed on 17 patients, one patient having been studied 3 times. Of the 17 patients 16 were white and one was a Negro. There were 15 men and 2 women. The types of liver disease are shown in the tables. Seven patients exhibited evidence of hepatic encephalopathy at some time during their hospitalization. Two patients (E. W. and M. G.) were studied while exhibiting the neurologic changes of hepatic encephalopathy. Twelve patients exhibited marked palmar erythema and 5 had minimal or no palmar erythema.

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Arterial and antecubital venous ammonia determinations were performed in 10 patients and venous concentrations alone were determined in 7. Femoral vein ammonia determinations were performed in 3 patients. In 5 patients simultaneous oxygen and ammonia concentrations were determined.

In those patients in whom arterial blood was studied, an indwelling Cournand needle was placed in the brachial artery. Blood was drawn from an antecubital vein with minimal stasis before, during, and after exclusion of the palm from the circulation. Exclusion of the palm was accomplished by the use of a sphygmomanometer cuff placed around the wrist and inflated above systolic pressure for at least one minute prior to sampling. The postexclusion samples were obtained from 5 to 15 minutes after the return of the hand to the circulation.

Blood ammonia was determined by a modification of the method of Conway[6] using a 10-minute diffusion period begun 3 minutes after the blood was drawn. Blood was placed in a bottle previously washed with carbon dioxide and containing potassium oxalate. The normal value, in venous blood, by this method is 31 mcg. NH_3-N_2 (s.d. ± 11) per 100 ml.

Oxygen content and capacity were determined by the method of Van Slyke and Neill.[7]

RESULTS

Because of the small sample and the wide range of overlap of the results in the two groups of patients, valid comparison of the A-V difference in ammonia before exclusion could not be made. Although not statistically significant the average A-V difference was smaller in those patients with palmar erythema (15 mcg. per 100 ml.) than in those without erythema (23 mcg. per 100 ml.).

A marked decrease in the venous ammonia concentration occurred with exclusion of the palm from the circulation in all except one (A. S.) of the patients with palmar erythema. The average exclusion values were 47 per cent (range 27.5 to 75.9 per cent) of the pre-exclusion levels (Table I). Upon return of the palm to the circulation the average ammonia concentration equaled 100 per cent (range 57.9 to 161.2 per cent) of the control values.

One patient with palmar erythema (A. S.) showed an increase in ammonia concentration upon exclusion of the palm from the circulation. These results were confirmed by repeating the study the same day. Several months later, however, when the same procedure was repeated, a significant fall in ammonia concentration occurred with exclusion of the palm (Table I).

In the 3 patients with erythema in whom oxygen saturations were studied, exclusion of the palm resulted in a significant fall in venous saturation and an increased arteriovenous difference in oxygen saturation confirming the presence of an arteriovenous shunt (Table II).

In 2 of the 3 patients in whom femoral vein ammonia determinations were obtained, the ammonia concentration was identical with the antecubital vein concentration during exclusion of the palm. In the third patient, the femoral vein concentration was midway between the antecubital vein concentrations prior to and during exclusion (Table III).

In the 5 patients with minimal or no palmar erythema no change occurred in the blood ammonia concentration with exclusion of the palm from the circulation (Table I). In one patient, however, the postexclusion ammonia was markedly increased above the control and exclusion values.

Study of oxygen saturations in two of the patients in this group failed to reveal a decrease in venous oxygen saturations with exclusion of the palm (Table II).

TABLE I. ARTERIAL AND VENOUS BLOOD AMMONIA PRIOR TO, DURING, AND AFTER EXCLUSION OF THE PALM FROM THE CIRCULATION (NH_3-N_2 MCG./100 ML.)

PATIENT	DIAGNOSIS	PRIOR TO EXCLUSION		A-V DIFFERENCE	DURING EXCLUSION		A-V DIFFERENCE	AFTER EXCLUSION		A-V DIFFERENCE
		BRACHIAL ARTERY	ANTECUBITAL VEIN		BRACHIAL ARTERY	ANTECUBITAL VEIN		BRACHIAL ARTERY	ANTECUBITAL VEIN	
Patients With Palmar Erythema										
B. F.	Cirrhosis	91	85	6	85	24	61	81	74	7
B. Y.	Alcoholic pellagra	56	44	12	52	34	18	–	42	–
C. P.	Cirrhosis	74	81	–7	78	32	46	66	63	3
R. L.*	Cirrhosis	–	50	–	50	19	31	49	59	–10
W. L.	Metastatic disease of liver	78	38	40	75	16	59	75	48	18
M. P.*	Cirrhosis	68	27	41	63	12	51	56	44	12
A. W.	Cirrhosis	–	79	–	–	40	–	–	59	–
S. B.	Cirrhosis	–	93	–	–	58	–	–	94	–
S. G.	Cirrhosis	–	69	–	–	30	–	–	40	–
A. S.*—A.M.	Cirrhosis	–	92	–	–	125	–	–	–	–
P.M.		–	96	–	–	148	–	–	131	–
2 months later		–	154	–	–	99	–	–	110	–
P. C.*	Cirrhosis hepatoma	–	111	–	–	81	–	–	–	–
M. G.	Cirrhosis	–	121	–	–	48	–	–	–	–
Patients With Minimal or No Erythema										
E. A.	Cirrhosis	46	34	12	49	39	10	41	37	4
E. T.	Chlorpromazine jaundice	–	36	–	75	42	33	80	74	6
A. G.*	Cirrhosis	69	25	44	–	22	–	–	–	–
R. S.	Cirrhosis	53†	54	–1	–	48	–	–	–	–
E. W.	Cirrhosis	–	100	–	–	113	–	–	98	–

*Previously showed evidence of hepatic encephalopathy.
†Femoral artery.

TABLE II. OXYGEN AND BLOOD AMMONIA CONCENTRATIONS WITH EXCLUSION OF THE PALM FROM THE CIRCULATION

PATIENT	DIAGNOSIS	PRIOR TO EXCLUSION		A-V DIFFERENCE	DURING EXCLUSION		A-V DIFFERENCE	AFTER EXCLUSION		A-V DIFFERENCE
		BRACHIAL ARTERY	ANTECUBITAL VEIN		BRACHIAL ARTERY	ANTECUBITAL VEIN		BRACHIAL ARTERY	ANTECUBITAL VEIN	
		Patients With Palmar Erythema								
B. Y.	Alcoholic pellagra									
	NH_3–N_2 mcg./100 ml.	56	44	12	52	34	18	–	42	–
	O_2 saturation	89%	77%	12	88%	41%	47	–	–	–
C. P.	Cirrhosis									
	NH_3–N_2 mcg./100 ml.	74	81	–7	78	32	46	66	63	3
	O_2 saturation	91%	57%	34	89%	44%	45	–	–	–
S. G.	Cirrhosis									
	NH_3–N_2 mcg./100 ml.	–	69	–	–	30	–	–	40	–
	O_2 saturation	94%	85%	9	93%	37%	56	–	–	–
		Patients With Minimal or No Palmar Erythema								
E. M.	Cirrhosis									
	NH_3–N_2 mcg./100 ml.	46	34	12	49	39	10	41	37	4
	O_2 saturation	87%	58%	29	–	57%	–	–	–	–
R. S.	Cirrhosis									
	NH_3–N_2 mcg./100 ml.	53	54	–1	–	48	–	–	–	–
	O_2 saturation	90%	48%	42	–	45%	–	–	–	–

TABLE III. COMPARISON OF ANTECUBITAL AND FEMORAL VEIN AMMONIA CONCENTRATION (NH_3–N_2 MCG./100 ML.)

PATIENT	DIAGNOSIS	ANTECUBITAL VEIN		FEMORAL VEIN
		PRIOR TO EXCLUSION	DURING EXCLUSION	
A. W.	Cirrhosis	79	40	40
P. C.	Cirrhosis hepatoma	111	81	82
M. G.	Cirrhosis	121	48	87

DISCUSSION

The decrease in ammonia concentrations observed with exclusion of the palm may be attributed to either of two mechanisms: (1) an effect of oxygen or other characteristics of arterial blood upon the ammonia determination or (2) the exclusion of the arteriovenous shunts may allow for more accurate reflection of tissue metabolism by antecubital venous blood. If the observed changes in ammonia are due to the effect of oxygen it should be noted that a linear correlation with the changes in oxygen saturation has not been observed (Table II).

The observation that femoral vein ammonia concentrations were lower than antecubital concentrations indicates that in the presence of palmar erythema the antecubital vein ammonia may be erroneously high. This is substantiated by the fact that with exclusion of the palm antecubital venous ammonia concentrations decreased significantly and were similar to those in the femoral vein.

The rise in blood ammonia after removal of the cuff which occurred in patient E. T. cannot be explained unless a technical error in the ammonia determination occurred in either the pre- or postexclusion determination. It would appear unlikely that the exclusion itself produced tissue changes resulting in ammonia production or that reflex vasodilation occurred in view of the fact that such a postexclusion rise was not regularly seen in other patients.

The results observed in one patient who demonstrated a rise in ammonia with exclusion of the palm on two separate sets of determinations in one day and subsequently, several months later, demonstrated a fall are difficult to explain except on the basis of technical error or by hypothesizing that the tissue metabolism changed between the two sets of studies. These studies were performed at room temperature; it is possible that extremes of temperature may alter these results.

Despite the conflicting interpretations of arteriovenous differences by various investigators, the presence or absence of palmar erythema appears to be of significance in studies of ammonia metabolism.

SUMMARY

Arterial and antecubital venous blood ammonia determinations were performed in patients with liver disease prior to, during, and after exclusion of the palm from the circulation.

A significant decrease in the ammonia concentration of antecubital venous blood occurred with exclusion of erythematous palms from the circulation but

no change was observed in patients without palmar erythema. Antecubital venous oxygen concentrations were similarly altered by exclusion of the erythematous palm.

The presence of arteriovenous anastomoses in palmar erythema has been confirmed. The presence of such shunts may introduce an error in studies of other tissue metabolites when arterial-antecubital vein determinations are used.

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SITES OF ORIGIN AND REMOVAL OF BLOOD AMMONIA FORMED DURING GLYCINE INFUSION: EFFECT OF L-ARGININE

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INTRODUCTION

THE more widespread use of amino acids in parenteral nutrition and clinical investigation has resulted in renewed interest in the toxicity of these compounds. One form of toxicity, evidenced by vomiting, convulsions, coma, or death, occurs when certain naturally occurring amino acids are administered parenterally to fasted animals or man, and is associated with a rise in the blood ammonia concentration.[1-4]

While studying the toxicity of certain L-amino acid mixtures in rats, Greenstein and co-workers[1, 2] observed that L-arginine protected against the acute toxic effects of these mixtures and prevented the associated blood ammonia rise. Najarian and Harper[5] demonstrated the effectiveness of L-arginine in correcting the blood ammonia elevation due to glycine infusion in dogs. In similar observations in man, Fahey[4] showed that L-arginine prevented the blood ammonia rise resulting from intravenous administration of glycine or of an arginine-deficient L-amino acid mixture.

The efficacy of L-arginine in preventing or correcting the ammonia rise due to amino acid administration suggests its use in other clinical situations associated with elevated blood ammonia levels. It would be helpful in assessing the clinical usefulness of arginine to know (1) where ammonia is released into the circulation and where it is removed during amino acid infusion, (2) in what organ(s) arginine acts to lower the blood ammonia, and (3) whether arginine causes increased ammonia removal from the blood or diminished ammonia release. The present study in dogs was undertaken to answer these questions, using glycine as the administered amino acid.

MATERIALS AND METHODS

Amino Acids.—Glycine was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. L-Arginine, which was used as the monohydrochloride, was obtained from Mann Research Laboratories, Inc., New York, N. Y. Each was free from other amino acids as demonstrated by paper chromatography.

Animals.—Adult mongrel dogs of both sexes, weighing 9 to 14 kilograms, were used. They were fed "Red Heart" dog food sufficient to maintain weight, but food was withheld for 24 to 36 hours prior to each experiment. The animals were under sodium pentobarbital anesthesia throughout the experimental period.

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Ammonia Determination.—Ammonia was determined in duplicate by the method of Seligson[6] using heparinized whole blood immediately after it was withdrawn.[4]

Procedures.—Hepatic vein and pulmonary artery catheterizations were performed through the jugular vein under fluoroscopic control. In the initial studies, the position of the hepatic vein catheter was confirmed at autopsy or by demonstrating a significant arteriovenous Bromsulphalein difference after the intravenous injection of this dye.[7] The renal vein catheters were introduced through a femoral vein and positioned at laparotomy. Portal vein blood was obtained by direct puncture. Blood from peripheral vessels (femoral artery, femoral vein, jugular vein) was taken through indwelling Cournand needles. The procedure for hepatectomy was a modification of that first described by Firor and Stinson,[8] and consisted of total removal of the liver after the segment of inferior vena cava passing through the liver was replaced with a siliconized glass tube. In one group of experiments the portal vein was anastomosed to the sidearm of the tube; in another group, the portal bed organs were removed. The hepatectomized animals received 5 per cent dextrose in water intravenously throughout the study. Nephrectomy was performed by ligating the renal pedicles and excising both kidneys.

Experimental Design.—In each experiment, following the particular surgical procedure and/or catheterizations, glycine was administered intravenously at a constant rate of 0.25 mM (19 mg.) per kilogram per minute for approximately 90 minutes, using a Bowman infusion pump. About 60 minutes after the start of glycine, L-arginine was injected intravenously within one minute in a total dose of 1.2 to 1.4 mM (250 to 290 mg.) per kilogram (approximately 6 per cent of the total moles of glycine). Blood ammonia concentration was determined nearly simultaneously in various vessels at intervals during each experiment. As will be noted, in one group of experiments L-arginine was given together with the glycine.

Calculation of Venous-arterial Ammonia Differences.—Most of the results will be expressed in terms of the venous-arterial differences in ammonia concentration across a given organ or tissue. This value was obtained by subtracting the arterial (femoral) blood ammonia concentration from the ammonia concentration in the venous blood draining the particular organ. A positive value indicates ammonia release; a negative value, ammonia removal. In the case of the liver, which has a dual blood supply, the venous-arterial (V-A) difference used was the hepatic vein-arterial difference unless explicitly noted. The V-A difference across the lung was obtained by subtracting the pulmonary arterial ammonia concentration from the ammonia concentration in the femoral artery.

RESULTS

Normal Fasted Dogs.—The arterial ammonia concentration of fasted anesthetized dogs varied between 1.0 and 1.8 μg of ammonia nitrogen per milliliter of whole blood. The average of 34 observations in 15 dogs was 1.40 ± 0.22 (s.d.) μg NH_3-N/ml. Renal and portal vein blood had a higher ammonia concentration than arterial blood (Table I, *B* and *C*). The ammonia concentration of hepatic vein blood was considerably lower than that in the portal vein and usually slightly less than the arterial level (Table I, *A*). There was no consistent venous-arterial difference in the limbs, head, or lungs (Table I, *D*, *E*, and *F*).

Blood Ammonia Changes With Glycine Infusion.—Ten experiments were performed in 8 dogs, and the results are presented in terms of venous-arterial ammonia differences in Table I. Two experiments are presented in more detail in Figs. 1 and 2. During intravenous glycine administration to the normal fasted animals, additional ammonia was added to the blood in the liver and kidneys (positive V-A difference, Fig. 1, *B* and *C*, and Table I). That the ammonia came from the liver itself and not from the portal bed is shown by the experiment illustrated in Fig. 2, in which the hepatic vein blood ammonia

concentration exceeded both the arterial and portal vein levels (Fig. 2, *A*). Also, the portal vein-arterial ammonia difference did not increase when glycine was given (Fig. 2, *C* and Table I, *C*).

As the arterial ammonia concentration increased during glycine infusion, ammonia was removed in the head and in the limbs. There was little change in ammonia concentration as the blood traversed the lungs (Table I).

TABLE I. VENOUS-ARTERIAL AMMONIA DIFFERENCES ACROSS THE LIVER, KIDNEY, PORTAL BED, LIMBS, HEAD, AND LUNGS BEFORE AND DURING GLYCINE INFUSION AND AFTER L-ARGININE INJECTION

DOG NO.	V-A NH_3 DIFFERENCE* (μg NH_3-N/ML. BLOOD)			MAXIMUM ARTERIAL NH_3†
	BEFORE GLYCINE	DURING GLYCINE	20 MIN. AFTER ARGININE	
A. Liver				
11a	−0.40	+1.2	−1.2	9.4
16a	+0.05	+6.8	−2.2	12.0
18	−0.30	+5.1	−4.5	25.0
23	−0.15	+3.0	−1.6	32.0
22	−0.25	+5.5	−11.0	37.0
	−1.4‡	+4.3‡	−12.0‡	
21§	−0.40	−0.6	−1.5	4.8
	−2.4‡	−1.8‡	−3.3‡	
11b	−0.05	−0.2	−1.5	3.7
16b	−0.05			
15	+0.10			
B. Kidney				
11a	+0.9	+4.2	+5.5	9.4
7	+1.2	+4.3	+5.5	17.0
18	+1.7	+3.5	+4.8	25.0
23	+2.0	+3.1	+6.0	32.0
16b	+1.0			
19	+1.1			
20	+1.7			
C. Portal Bed				
22	+1.2	+0.8	+1.1	37.0
21§	+2.0	+1.9	+2.1	4.8
D. Limb				
11a	−0.20	−2.3	−0.30	9.4
16a	−0.10	−2.4	+0.80	12.0
18	0.0	−2.2	+0.60	25.0
23	−0.40	−17.0	+0.20	32.0
7	+0.10			
16b	+0.05			
19	0.0			
20	−0.30			
25	+0.05			
E. Head				
21§	+0.30	−1.5	−0.50	4.8
29	−0.20	−1.0	−0.20	
27	−0.20			
28	+0.30			
F. Lungs				
29	0.0	−0.25	−0.10	
30	+0.15	+0.20	−0.0	7.5
28	−0.20			

*A positive value indicates ammonia release by the organ; a negative value, ammonia removal.

†During glycine infusion.

‡Hepatic vein − Portal vein NH_3 difference.

§Rate of glycine infusion 0.18 mM/Kg./min.

Effect of L-*Arginine.*—A 60-minute infusion of glycine, without injection of arginine, caused a marked rise in blood ammonia concentration, which continued to rise after cessation of the infusion and resulted in the death of the animals. When L-arginine (1.2 to 1.4 mM/Kg.) was injected intravenously during the glycine infusion, the arterial ammonia concentration fell rapidly, and concomitantly there was abrupt cessation of ammonia release by the liver followed by net removal of ammonia in this organ (Figs. 1 and 2 and Table

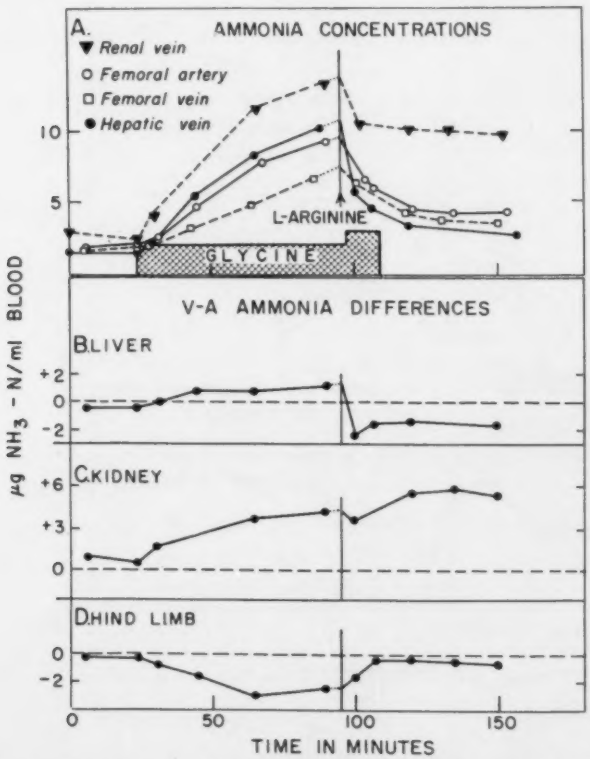

Fig. 1.—Changes in blood ammonia concentrations and venous-arterial differences across the liver, kidney, and hind limb in Dog 11a during glycine infusion, before and after L-arginine administration.

I, *A*). These animals survived with no apparent ill effect. Arginine did not affect the V-A ammonia difference of the kidneys, portal bed, or lungs. The changes in limb and head V-A ammonia differences noted in Fig. 1, *D* and Table I, *D* and *E*, are probably related to the fall in arterial ammonia concentration and not specifically to arginine administration. Similar changes in peripheral V-A ammonia difference accompany the fall in arterial ammonia concentration at the end of an infusion of ammonium salts.[9]

In two experiments, including one in which the rate of glycine administration was less than 0.25 mM/Kg./min., the hepatic vein blood ammonia concentration never exceeded the arterial level (Table I, *A*, Dogs 21 and 11b). In each of these instances the arterial concentration rose only moderately, and arginine caused only a slight drop in the arterial ammonia concentration (from 4.8 to 4.0 μg NH_3-N/ml. in Dog 21 and from 3.7 to 2.8 in Dog 11b). The results of these experiments may be explained by assuming that there was insufficient depletion of arginine during the period of fasting.

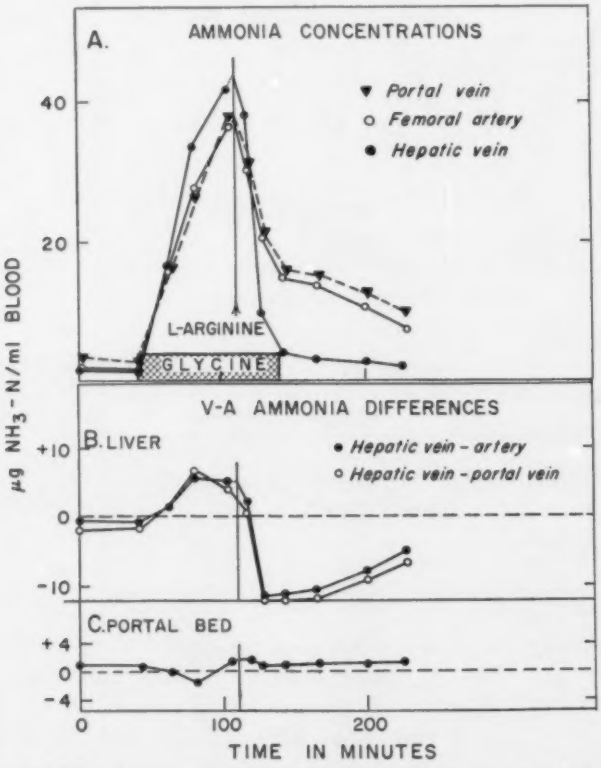

Fig. 2.—Changes in blood ammonia concentrations and venous-arterial differences across the liver and portal bed in Dog 22.

When L-arginine was infused simultaneously with glycine, the liver removed ammonia from the blood continuously, and the arterial ammonia concentration increased only slightly (Fig. 3). The renal V-A ammonia difference was essentially the same as when glycine was given alone, again indicating that arginine does not affect renal ammonia production during glycine infusion.

Effect of Hepatectomy.—Five hepatectomized dogs were studied. After hepatectomy an elevation of the arterial blood ammonia concentration was evident. This was greater when the gastrointestinal tract was intact (portacaval

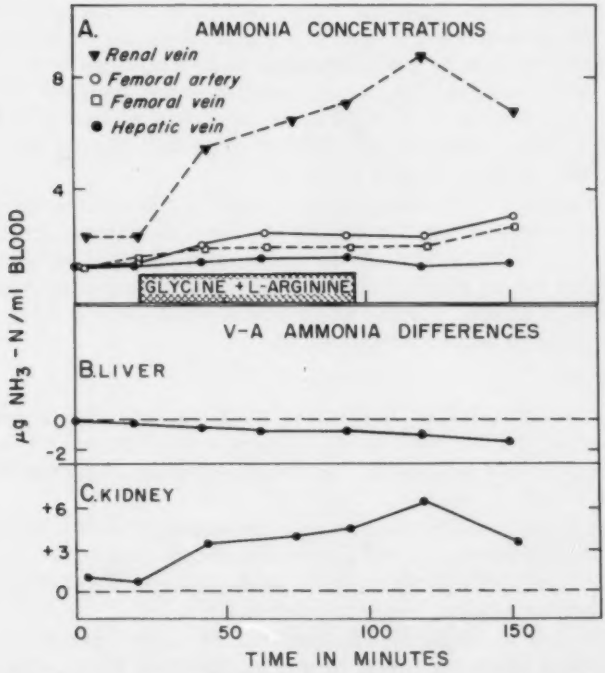

Fig. 3.—Blood ammonia changes when L-arginine is infused simultaneously with glycine.

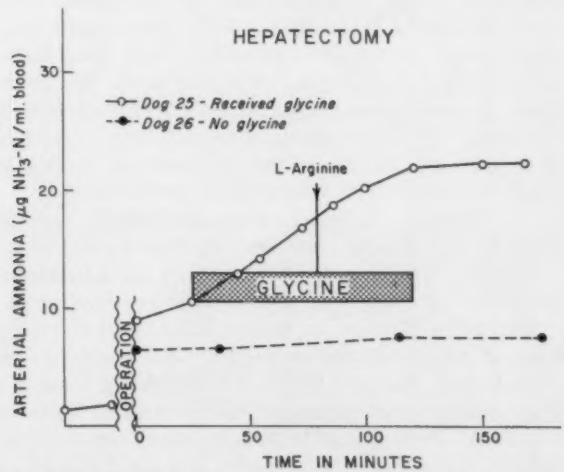

Fig. 4.—The effect of L-arginine on the arterial ammonia concentration during glycine infusion in the hepatectomized dog. The lower curve represents a hepatectomized animal which received no glycine. In each case a portacaval shunt was done, leaving the portal bed organs intact.

shunt) than when the portal bed organs were removed, an observation consistent with the previous demonstration of ammonia release from the intestinal tract. When glycine was infused into two of these animals, a further rise in the arterial ammonia level ensued and the injection of L-arginine had no effect (Fig. 4). Of note is the fact that the renal V-A ammonia difference increased during glycine infusion even in the absence of the liver (from 0.7 to 3.5 μg NH_3-N/ml. in Dog 31 and from 0.6 to 8.5 in Dog 19).

Effect of Bilateral Nephrectomy.—Nephrectomy had no acute effect on the ammonia concentration of arterial or hepatic vein blood in the two dogs studied. Ammonia release in the liver and marked elevation of the arterial ammonia concentration occurred during glycine infusion. As in the intact animal, injection of L-arginine caused abrupt cessation of ammonia release by the liver and a rapid fall in the arterial ammonia level.

DISCUSSION

Ammonia Production.—The observations in the normal fasted dog that ammonia is released into the blood by the kidneys and portal bed organs and removed in the liver are in accord with those previously reported for the dog[10, 11] and for man.[12, 13]

Hepatic ammonia production following intravenous injections of glycine has been demonstrated by Bornstein,[14] and renal ammonia production, by Polonovski and associates.[15] The present study confirms these observations with the use of different chemical and experimental methods.

Whether the liver or the kidney is quantitatively more important in ammonia release during glycine infusion cannot be answered directly from the results of the present study, since blood flow measurements were not done. However, the absence of the usual marked rise in the arterial ammonia concentration whenever the liver failed to release ammonia suggests that the liver is probably the major source of the excess ammonia in the blood during glycine infusion. This is further supported by a comparison of the rise in arterial ammonia concentration in the nephrectomized animals with that observed in the hepatectomized animals. The latter group, with only the kidneys to produce ammonia, showed a much smaller rise in arterial ammonia during glycine infusion than did the nephrectomized animals with livers intact.

When glycine is infused, the increased blood ammonia may be derived directly from glycine by deamination, indirectly by deamination of an intermediate compound, or from nitrogen sources other than the administered amino acid. In favor of direct deamination of glycine as the mechanism of ammonia production is the correlation between the known presence of glycine oxidase in the liver and kidneys of many mammalian species[16] and ammonia release solely by these organs. Although the activity of this enzyme in vitro is quite low at physiologic pH and substrate concentration,[16] in the presence of large amounts of glycine (for example, during intravenous infusion) its activity may be significant.[17]

The possibility that an intermediate between glycine and ammonia (e.g., glutamine) is first formed and that this intermediate compound is then deaminated has not been ruled out. However, renal ammonia release in the

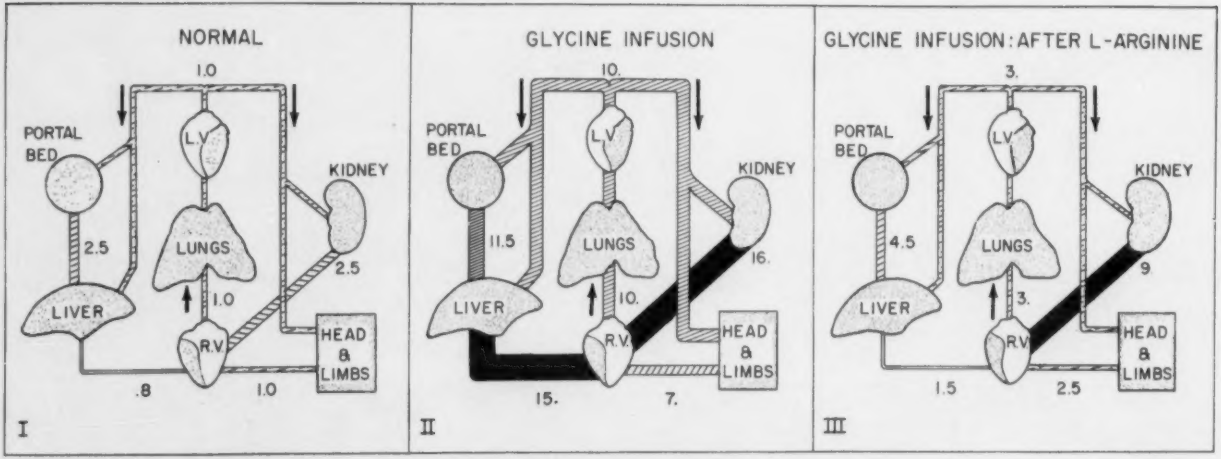

Fig. 5.—A diagrammatic summary of the blood ammonia changes during glycine infusion, and the effect of L-arginine. The figures represent the blood ammonia concentrations typically found (in μg NH_3-N/ml.); the higher the concentration, the darker and wider the lines. In the normal animal (I) ammonia is added to the blood in the kidneys and portal bed and removed in the liver. During glycine infusion (II) additional ammonia is released by the liver and kidneys. When L-arginine is injected during infusion of glycine (III), the liver no longer releases ammonia, but removes it from the incoming blood.

absence of the liver would indicate that in any case the liver is not needed for ammonia production by the kidney. Similarly, hepatic ammonia production is not dependent on the kidneys.

L-*Arginine activity:* Previous observations demonstrated that L-arginine would prevent or correct the blood ammonia rise and associated toxicity caused by intravenous infusion of glycine or certain other amino acids,[1, 2, 4] but did not indicate whether arginine caused (1) increased removal of ammonia from the blood or (2) diminished release of ammonia into the circulation. The results of the present study show that arginine acts both to curtail ammonia release and to accelerate ammonia removal, at least when glycine is the amino acid used. It acts solely in the liver and has no effect on ammonia release in the kidney as estimated by V-A differences.

The essential changes in ammonia formation and removal produced by arginine are presented diagrammatically in Fig. 5.

The hepatic site of arginine activity is consistent with its role in the Krebs-Henseleit urea cycle.[18] In the fasted dog receiving glycine a relative deficiency of arginine apparently limits the rate of conversion of ammonia to urea, so that some of the ammonia formed in the liver escapes into the blood. When arginine is supplied, urea synthesis is accelerated[2, 5, 20] and the liver no longer releases ammonia but actually removes it from the incoming blood.

In considering the potential usefulness of L-arginine in clinical states associated with elevated blood ammonia concentrations, it should be emphasized that one of the major effects of arginine in lowering the high blood ammonia due to glycine infusion is the prevention of ammonia release by the liver. However, in most clinical conditions where the blood ammonia is high, ammonia release by hepatic cells does not occur,[13, 19] but rather the elevated blood ammonia is due to addition of ammonia directly into the systemic circulation. This occurs, for example, in patients with extensive portal-systemic collaterals, allowing ammonia of intestinal origin to by-pass the liver, or in patients receiving ammonium salts intravenously. In these circumstances arginine can lower the blood ammonia only by accelerating ammonia removal in the liver. Whether such increased removal would be sufficient to lower significantly the high arterial ammonia concentrations in these clinical conditions is yet to be answered. Of note in this regard is the observation that in none of the present experiments did arginine return the arterial ammonia concentration to normal during the period of observation. Furthermore, in the two experiments in which the liver failed to release ammonia, arginine caused only a slight fall in the arterial ammonia concentration. These persistently elevated ammonia levels may have been due to continued release of ammonia into the systemic circulation by the kidneys.

The need for L-arginine (or related amino acids of the urea cycle) to prevent ammonia toxicity during amino acid administration in man has already been demonstrated,[4] and studies are now underway to determine its therapeutic value in other clinical states associated with an elevation in blood ammonia.

SUMMARY

In the normal fasted dog ammonia was added to the blood in the kidneys and portal bed and was removed in the liver.

Glycine given intravenously resulted in ammonia release into the blood in the liver and additional ammonia release by the kidneys, with concomitant rise in the arterial ammonia concentration. At high arterial levels ammonia was removed in the extremities and head.

L-Arginine injected intravenously during the glycine infusion produced an abrupt cessation of ammonia release by the liver and caused this organ to remove ammonia from the incoming blood. A rapid fall in arterial ammonia concentration occurred. Arginine did not affect ammonia release or removal by any organ tested other than the liver.

The possible therapeutic significance of the capacity of L-arginine to prevent ammonia release at an important site of ammonia formation is discussed.

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LABORATORY METHODS

FLOCCULATION TESTS FOR TULAREMIA

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THE agglutination test is one of the most useful and reliable methods for the diagnosis of tularemia. Francis and Evans[1] reported on a study of the agglutination reaction in tularemia. Also, Ransmeier and Ewing[2] reported on the presence of agglutinins in 87 nonfatal cases and 10 fatal cases of tularemia. It was found that the variation in turbidity of antigen exerted some effect on the titer of antitularensis and antiabortus serums. Wright and Feinberg[3] have studied the reaction of a polysaccharide of *Pasteurella tularensis* with erythrocytes, the agglutination of sensitized cells by antiserum, and the inhibition of agglutination by free polysaccharide.

In previous reports on the flocculation test for brucellosis, Hunter and Colbert[4, 5] have shown that an antigen consisting of cholesterol, lecithin, and brucella extract could be standardized to a definite specificity and sensitivity and that tests were reproducible. Furthermore, the tests were rapidly performed and very easily read. Therefore, it seemed reasonable that tularensis flocculation antigen and tests could be developed. This paper reports the results of this work.

MATERIAL AND METHODS

Preparation of Antigen Extract.—The tularemia antigen was prepared by growing *P. tularensis* (No. 38 NIH) on Bacto-Cystine heart agar plus 5 per cent sheep blood for 48 hours at 35° C. The organisms were washed off with 0.85 per cent saline containing 0.25 per cent phenol. Additional phenol was added so that final concentration was 2 per cent. The suspension of organisms was allowed to stand in the refrigerator at approximately 8° C. for 10 days. It was then dialyzed against running tap water for 72 hours or until phenol free, using Dupont No. 300-A cellophane. The suspension was centrifuged at 2,500 r.p.m. for 30 minutes, the supernate was made isotonic by the addition of NaCl, and Merthiolate was added to make a 1:10,000 dilution. This tularensis extract was kept either in a Deep-freeze or in the refrigerator.

Lecithin: Lecithin (ex-beef) in absolute alcohol was diluted in absolute ethyl alcohol so that the amount of lecithin was 10 mg. per milliliter or a 1 per cent solution.

Cholesterol: This was a 1 per cent cholesterol (ash free, precipitated from alcohol for Kline test) solution in absolute alcohol.

Other reagents: Distilled water (approximately pH 6.0); 0.85 per cent solution of NaCl (Mallinkrodt, AR); 1 per cent solution of NaCl (Mallinkrodt, AR); and Eagle's buffered saline, pH 7.4.

Apparatus: The equipment necessary to perform these tests is found in almost any laboratory performing the serologic tests for syphilis.

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Preparation of Serum.—The serum was separated from the clots by centrifuging and pipetting or decanting.

The serum was heated at 56° C. in a water bath for 15 minutes. Serum to be rerun the following day should be reheated for 5 minutes at 56° C.

Preparation of Antigen Emulsion.—To the bottom of a 30 ml. round glass stoppered bottle having a flat bottom (Corning 90530, stopper No. 14) 0.85 ml. of distilled water (approximately pH 6.0) was pipetted. While rotating the bottle, 1 ml. of 1 per cent cholesterol was added, drop by drop, and rotation was continued for 20 seconds. Then 0.1 to 2.5 ml. of 1 per cent lecithin was added and shaken vigorously for 15 seconds. (The exact amount of lecithin to use must be determined by standardization according to method discussed later. In most instances the amount of lecithin will be 0.15 ml. Titration is necessary for each new lot of lecithin and/or tularensis extract.) A predetermined dose (0.05 to 0.2 ml.) of tularensis extract was added and shaken vigorously by striking the bottom of the bottle against the palm of the hand for 1 minute. (The amount or dose of tularensis extract to use must be determined for each lot of extract. The method of standardization is presented later.) Finally, 2.5 ml. of Eagle's buffered saline was added and shaken less vigorously for 30 seconds. The antigen emulsion is usable immediately and if refrigerated may be used for 24 hours. It is recommended, however, that a fresh antigen emulsion be made up and used the same day.

Standardization of Antigen.—

Standard antigen: The level of sensitivity and specificity for a standard antigen was ascertained by comparing the results of the flocculation test with the agglutination test on several hundred human sera. All flocculation antigens were then titrated so that the results were comparable to the standard. The flocculation antigen is the only tularensis antigen thus far standardized to a definite sensitivity and specificity.

Method of standardizing antigen: In the preparation of antigens for the various flocculation tests for syphilis, the authors have found that experiments must be conducted to determine the optimal ratio of reagents used. Antigens must be prepared using various ratios and concentrations of cardiolipin, lecithin, and cholesterol so that the antigen will give the same sensitivity and specificity as the standard. The same is true in standardizing the tularensis antigen, the only difference being that tularensis extract is used in place of cardiolipin. The standardization was done by making 30 antigen emulsions by mixing each of 0.05, 0.1, 0.15, 0.2, and 0.3 ml. of tularensis extract with 0.0, 0.05, 0.1, 0.15, 0.2, and 0.25 ml., respectively, of 1 per cent lecithin.

The microscopic slide and tube flocculation tests were run on at least 10 sera of various reactivity with each of the 30 antigen emulsions. The ratio of tularensis extract and lecithin giving the same sensitivity and specificity as the standard antigen emulsion was determined. Once this ratio was determined for a particular lot of tularensis extract, it could be used until this lot of extract was exhausted. In other words, it is necessary to standardize each lot of tularensis extract only once.

Complete Antigen.—In order to simplify the preparation of the antigen emulsion it was possible to combine the cholesterol, lecithin, and tularensis extract into one reagent. After standardizing the antigen according to directions previously described, then the combination could be carried out. For example, if the composition of antigen emulsion was 1 ml. of 1 per cent cholesterol, 0.15 ml. of 1 per cent lecithin, and 0.10 ml. of tularensis extract, then the combined antigen would contain 100 ml. of 1 per cent cholesterol, 15 ml. of 1 per cent lecithin, and 10 ml. of tularensis extract. The lecithin was added to cholesterol, mixing well, and the tularensis extract added slowly with adequate shaking. The dose of this completed antigen would be 1.25 ml. It should be stored at room temperature. Preparation of the antigen emulsion with complete antigen was as follows: To the bottom of a round glass stoppered bottle having a flat bottom (Corning 90530, stopper No. 14) 0.85 ml. of distilled water (approximately pH 6.0) was pipetted. While rotating 1.25 ml. (1 dose) of complete tularensis antigen was added, drop by drop, and then shaken vigorously for 15 seconds. Finally, 2.5 ml. of Eagle's buffered saline was added and shaken less vigorously for 10 seconds.

TABLE I. COMPARISON OF THE AGGLUTINATION TEST WITH THE FLOCCULATION TEST FOR TULAREMIA AND THE BRUCELLA FLOCCULATION TEST

SPECIMEN NUMBER	P. TULARENSIS AGGLUTINATION TEST								P. TULARENSIS TUBE FLOCCULATION TEST								BR. ABORTUS FLOCCULATION TEST						
	DILUTIONS								DILUTIONS								DILUTIONS						
	20	40	80	160	320	640	1,280	2,560	20	40	80	160	320	640	1,280	2,560	20	40	80	160	320	640	1,280
434	4	4	4	4	1	-			P	P	P	P	P	WP	N		N	N	N	N	N	N	
3,189	4	4	2	-	-	-			P	P	P	WP	N	N			N	N	N	N	N	N	
3,205	1	-	-	-	-	-			P	P	WP	N	N	N			N	N	N	N	N	N	
3,364	4	3	1	-	-	-			P	P	WP	WP	N	N			P	N	N	N	N	N	
3,531	-	-	-	-	-	-			P	P	WP	N	N	N			N	N	N	N	N	N	
3,550	4	4	3	2	1	-			P	P	P	P	P	WP	N		P	N	N	N	N	N	
3,955	4	4	3	1	-	-			P	P	P	WP	N	N			N	N	N	N	N	N	
2,632	2	2	4	4	4	3	1	-	P	P	P	P	P	P	P	N	N	N	N	N	N	N	
2,161	2	1	-	-	-	-			P	P	P	WP	N	N			N	N	N	N	N	N	
4,303	4	4	4	4	3	2	-		P	P	P	P	P	P	WP	WP	P	WP	N	N	N	N	
4,655	4	4	4	1	1	-			P	P	P	P	WP	N			N	N	N	N	N	N	
4,377	4	4	2	1	-	-			P	P	P	P	WP	WP	N		N	N	N	N	N	N	
4,517	4	4	4	4	4	2	-	-	P	P	P	P	P	WP	WP	N	N	N	N	N	N	N	
4,774	4	4	3	2	-	-			P	P	P	P	WP	N	N		N	N	N	N	N	N	
4,763	4	4	2	-	-	-			P	P	P	WP	N	N			N	N	N	N	N	N	
4,774	4	4	3	2	-	-			P	P	P	P	WP	N			N	N	N	N	N	N	
7,046	4	4	2	-	-	-			P	P	P	P	WP	N			N	N	N	N	N	N	
9,725	4	4	4	2	-	-			P	P	P	P	P	WP	N		N	N	N	N	N	N	
10,124	4	4	4	3	1	-			P	P	P	P	P	WP	N		N	N	N	N	N	N	
10,549	4	2	1	-	-	-			P	P	P	P	WP	N			N	N	N	N	N	N	

P = positive; WP = weakly positive; N = negative.

Microscopic Slide Test.—It is recommended that twofold dilutions of serum be made ranging from 1:10 to 1:640. Into paraffin or permanent ring glass slides 0.05 ml. of the serum dilutions were pipetted. One drop of antigen emulsion was added to each serum dilution using an 18 gauge needle, with bevel removed, held perpendicularly. This needle should deliver 80 to 85 drops of antigen emulsion. The slides were rotated for 4 minutes on a mechanical rotator at a speed of 180 r.p.m. and read immediately at a magnification of 100. Positive and negative serum and saline controls were always run. The reactions were recorded as follows: positive, P (+), large and medium sized clumps; weakly positive, WP (±), small clumps; negative, N (-), no clumping.

The titer of the serum obtained by the micoscopic slide test will not be comparable to that obtained by the tube test or by the agglutination test. The ratio of the serum (0.05 ml.) to the antigen (0.0125 ml.) is 4:1, while in the tube test it is 1:1.

Macroscopic Tube Test.—

Preparation of diluted antigen emulsion: The antigen emulsion as prepared for the microscopic slide test should stand for at least 1 hour at room temperature before diluting 1:5 with 1 per cent sodium chloride solution. This diluted antigen must stand for at least 5 minutes before use.

Procedure for performing the macroscopic tube test: Twofold dilutions of serum were made in 0.85 per cent NaCl ranging from 1:10 to 1:640. The volume of each dilution in the test tube (13 × 100) was 0.5 ml. To each tube 0.5 ml. of diluted antigen was added. The final dilutions then ranged from 1:20 to 1:1,280. The tubes were shaken on a Kahn shaker for 5 minutes and then centrifuged for 10 minutes at 1000 × G which was obtained by using a No. 2 International Equipment Company centrifuge at 2,000 r.p.m. with No. 925 head and No. 926 rack. Then the tubes were shaken on a Kahn shaker for exactly 1 minute and the tests were read immediately by holding in front of a reading lamp with black background. The results were recorded as follows: positive, P (+), large clumps and clear or slightly turbid medium; weakly positive, WP (±), small clumps in a cloudy medium; negative, N (-), no clumps and uniform turbidity which gives a swirl similar to that seen in a mixture of blood and saline.

Agglutination Test.—The agglutination test and the tube flocculation test were performed on approximately 2,500 human sera. The agglutination test was performed using 0.5 ml. of the diluted serum plus 0.5 ml. bacterial antigen. The bacterial antigen was prepared by using *P. tularensis* (No. 38 NIH) grown on Bacto-Cystine heart agar with 5 per cent sheep blood. After incubation at 35° C. for 48 hours the growth was washed off in 0.5 per cent formalin. The turbidity of the suspension was standardized by the Coleman spectrophotometer to give 50 per cent transmittance at a wave length of 600 mμ. The tests were incubated in the water bath for 3 to 4 hours at 50° C. and refrigerated overnight.

RESULTS

The results of the agglutination test and the flocculation tube test on 20 sera, which are representative of approximately 100 sera, are given in Table I. Also, included in this table are the results of the brucella flocculation test. It can be noted that the flocculation test gave a higher titer than the agglutination test. Furthermore, the results with the flocculation test were clear-cut, much easier to read, and were reproducible. Francis and Evans,[1] and Ransmeier and Ewing[2] have reported on the cross agglutination of tularemia sera with brucella antigens. From the results given in Table I it will be noted that only 3 out of 30 sera gave a reaction in low dilutions with the brucella flocculation test. Table II gives the results of the agglutination test and tube flocculation test with both *Brucella abortus* and *Pasteurella tularensis* rabbit immune sera using brucella and tularensis antigens. With the rabbit immune sera immunized with either *Br. abortus* extract or the whole organisms it will

TABLE II. ANTIGENIC CROSS REACTIONS BETWEEN P. TULARENSIS AND BR. ABORTUS

RABBIT ANTISERUM	BR. ABORTUS												P. TULARENSIS							
	TUBE FLOCCULATION TEST						TUBE AGGLUTINATION TEST						TUBE AGGLUTINATION TEST				TUBE FLOCCULATION TEST			
	DILUTIONS						DILUTIONS						DILUTIONS				DILUTIONS			
BR. ABORTUS	80	160	320	640	1,280	2,560	80	160	320	640	1,280	2,560	10	20	40	80	10	20	40	80
152*	P	P	P	P	WP	N	4	4	4	3	1	-	-	-	-	-	N	N	N	N
192*	P	P	P	P	WP	N	4	4	4	4	4	2	-	-	-	-	N	N	N	N
1,956*	P	P	P	P	P	WP	4	4	4	4	4	3	3	3	-	-	N	N	N	N
1,948	P	P	P	P	P	N	4	4	4	4	3	3	3	3	-	-	N	N	N	N
1,954	P	P	P	P	P	P	4	4	4	4	4	2	-	-	-	-	N	N	N	N

P. TULARENSIS	P. TULARENSIS												BR. ABORTUS							
	TUBE AGGLUTINATION TEST						TUBE FLOCCULATION TEST						TUBE AGGLUTINATION TEST				TUBE FLOCCULATION TEST			
	DILUTIONS						DILUTIONS						DILUTIONS				DILUTIONS			
P. TULARENSIS	80	160	320	640	1,280	2,560	80	160	320	640	1,280	2,560	10	20	40	80	10	20	40	80
48	4	4	3	1	-	-	P	P	P	P	WP	N	4	4	2	-	N	N	N	N
54	4	4	4	1	-	-	P	P	P	P	N	N	2	-	-	-	N	N	N	N

P = positive; WP = weakly positive; N = negative.
*Rabbits immunized with Brucella extract.

be noted that with the tularemia flocculation test there were no cross reactions. In the agglutination test for tularemia, two of the 5 sera did show some cross reaction. With the 2 rabbit sera immunized with *P. tularensis* the agglutination test for brucellosis gave some cross reaction while the brucella flocculation tests were negative.

TABLE III. ABSORPTION OF TULAREMIA IMMUNE SERUM WITH TULARENSIS ANTIGEN EMULSION AND TULARENSIS EXTRACT

	SERUM DILUTIONS						
Tularensis antigen emulsion	20	40	80	160	320	640	1,280
Before absorption	P	P	P	P	P	P	WP
First absorption		P	P	WP	N	N	N
Second absorption			N	N	N	N	N
Tularensis extract after first absorption	N	N	N	N	N	N	N

P = positive; WP = weakly positive; N = negative.

In order to determine if the tularensis extract was antigenically specific, experiments were carried out by absorbing tularensis antisera from rabbits with the tularensis extract. The results of these experiments are given in Table III. It will be noted that the agglutinins for tularensis were absorbed out with tularensis extract as well as with tularensis antigen emulsion. However, with the antigen emulsion the antiserum had to be absorbed twice. This is what one would expect since the amount of tularensis extract is present in very small amount.

DISCUSSION

The data present in this report have shown that an extract of *P. tularensis* can be adsorbed on cholesterol in the presence of lecithin to produce a flocculating antigen in which the sensitivity and specificity can be adjusted to the standard antigen. It has also been shown that the tests are reproducible and that no cross reaction occurs with brucella serum.

When a cross reaction between the agglutination or flocculation tests does occur in human serum it is difficult to determine whether the patient has previously had one or the other infection. An interesting case illustrating this statement occurred in our laboratories. In May, 1955, a serum was received from an individual with request by the physician that the agglutination tests be run. The results were a titer of 1:320 for tularemia and negative for brucella. Another specimen from the same patient was received in December, 1955, in which the titer for tularemia was 1:320 and for brucella 1:80. The clot was cultured and *Br. abortus* was isolated. Since *Br. abortus* was isolated from this patient in December it was concluded he had brucellosis. It is also suggestive that he had tularemia in May and later developed brucellosis. If the blood from this patient had been examined in December without isolating Brucella, it would no doubt have been interpreted that the individual had tularemia with some cross reaction with brucella.

During the fall and winter of 1956-1957 a very marked increase was noted in reactive bloods to the flocculation test for tularemia. In fact, the increase

was so great that our epidemiologist contacted the attending physicians of these cases and the laboratory results were confirmed with clinical findings. At the same time, the State Fish and Game Department reported that rabbits were dying and desired an investigation as to the cause. However, due to insufficient personnel in the State Board of Health it was not possible. Several years ago the rabbit population decreased very markedly and at that time *P. tularensis* was isolated from some sick and dead animals.

In developing the flocculation test for tularemia the many years experience in working out the tests for brucellosis was utilized. The preparation of a suitable extract of the organisms and establishment of a standard antigen was determined by comparison of the results of the flocculation test with the agglutination test on a large number of sera.

There were many reasons for using cholesterol instead of red cells or colloidin. One of the reasons being that it is not possible to standardize an antigen with either red cells or colloidin. With cholesterol a nonantigenic, purified chemical is used in a definite concentration thus producing a rather constant number of crystals. Furthermore, the experience of producing an antigen for the serology of syphilis in which the cholesterol is coated with cardiolipin in the presence of lecithin has proved successful in producing a test which is reproducible. This reproducibility is due to the fact that the antigen can be standardized to a definite sensitivity and specificity, the procedures of conducting the tests are controlled and the results very easily read. The experience of many years has shown that no other laboratory test has the reproducibility of the serologic tests for syphilis. A similar situation exists with the flocculation tests for brucellosis and for tularemia.

SUMMARY

1. Methods of producing a cholesterol, lecithin, turlarensis extract antigen emulsion for a microscopic slide and macroscopic tube flocculation test have been described.

2. The sensitivity and specificity of the antigen emulsion could be adjusted to conform to the standard antigen emulsion. Method of standardizing the antigen emulsion is described.

3. The procedure of making a complete antigen and the method of making the antigen emulsion using the complete antigen is given.

4. Data are presented showing the antigen emulsion to be specific.

5. The flocculation tests are rapidly performed, the results are clear-cut, and easily read. The tests give reproducible results and the titers are higher than with the agglutination test.

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A SIMPLE FLUID MEDIUM FOR DIPHASIC FUNGI

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THE increasing awareness of systemic mycoses has led to a widening study of their microbiologic properties. Such studies would be greatly facilitated by use of a culture medium which would allow long-term preservation of large numbers of strains. The simple fluid medium of Little and Subbarow[1] has been applied to the cultivation and preservation of the medically important diphasic fungi.

MATERIALS AND METHODS

The medium as described by Little and Subbarow[1] has been modified in common practice by the use of dried human blood-clot pellets rather than powdered rabbit blood. Human blood clots are dropped into boiling water for 15 min., rinsed in cold water to remove loose particles, drained, and stored in the refrigerator overnight. Pellets are cut ⅛ to ¼ inch thick, spread thinly on trays and dried at 37° C. The pellets should be agitated once or twice a day for 3 days to obtain even dehydration. The dried clots are then ready for use or may be stored without deterioration for as long as 2 years in tight screw-capped jars.

The broth component is prepared fresh of 2 per cent peptone, 0.5 per cent NaCl, and 0.2 per cent glucose in distilled water adjusted to pH 7.5.

The medium is combined in a ratio of 1 Gm. dry pellets to 30 or 40 ml. broth, and sterilized by autoclaving at 121° C. for 15 minutes.

RESULTS

The medium, dispensed in Army-type, rubber-stoppered vaccine bottles, was inoculated with pure yeast phase cultures of *Blastomyces dermatitidis* (12 strains), *Histoplasma capsulatum* (2 strains), and *Sporotrichum schenkii* (1 strain) in triplicate. Such preparations remained in viable yeast phase for one year when maintained at 37° C. A second group, after cultivation for 2 months at 37° C., was transferred to 20° C. for storage. These were all viable and in relatively smooth yeast phase after 2 years and 4 months. The third group of the same organisms, after 2 months at 37° C., was transferred to the refrigerator at 5° C. These were all viable at 18 months and in yeast phase, but quite rough.

In addition, conversion to yeast phase was regularly obtained from pure filamentous inoculum of these fungi. The conversion was slower for most strains than occurs on solid medium. However, two strains of *Blastomyces dermatitidis* which were difficult to convert and maintain in yeast phase on brain-heart blood

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agar, became smooth and stable after 4 months at 37° C. in the fluid medium. As with solid medium conversion, *Sporotrichum schenkii* was the slowest to convert in the fluid.

Recovery of cultures from the fluid medium was made repeatedly by sterile needle and syringe without disturbing the original stock preparation. A few drops of broth sprayed or streaked on brain-heart blood agar or Sabouraud's agar yielded isolated colonies of the phase desired. When larger amounts of inoculum were required, a quantity of the broth was centrifuged and the sediment used for seeding. The degree of smoothness of the recovered yeast phase varied with the strain, but all usually became creamy in 2 or 3 rapid transfers. The filamentous cultures also required 2 or 3 transfers before producing normal sporulation.

Some attempt was made to utilize adjuvants to increase the yield and smoothness of the yeast growth. The addition of cystine seemed to aid in the conversion of Histoplasma and Sporotrichum in this medium but did not influence the growth of Blastomyces. The addition of yeast extract improved the quantity and quality of the yeast phase growth of *B. dermatitidis*, but was detrimental to the growth of Histoplasma and Sporotrichum. Additives to the basic medium are unnecessary for routine preservation of strains.

Quantity growth of *B. dermatitidis* and *H. capsulatum* in this medium was tested by using a variation of the technique for growing *Trypanosoma cruzi*. The clot pellets and a portion of the broth were enclosed in a dialysis membrane to prevent contamination of the cells by clot fragments, and the membrane was suspended in the inoculated broth. A good yield of packed yeast cells was recovered by centrifugation of the broth portion at the end of 3 weeks' incubation at 37° C.

Other organisms, both mycotic and bacterial, can be maintained in this medium. *Cryptococcus neoformans*, Candida species, Nocardia species, and various Mycobacterium species grow well and are apparently stable.

SUMMARY

1. The clot-peptone broth in rubber-stoppered vaccine bottles permits preservation of yeast phase cultures of *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Sporotrichum schenkii* for at least 2 years without transfer.

2. The same organisms will convert from filamentous inoculum to yeast phase after extended incubation at 37° C.

3. Cultures can be recovered easily in either yeast phase or filamentous form without destroying the original stock preparation.

4. The medium is practical for the quantity production of yeast phase growth of these organisms, and may prove equally useful for the long-term preservation of other fungi and bacteria.

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MICROESTIMATION OF SERUM CHOLESTEROL AND ESTERS ON FINGER-TIP BLOOD

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THE need for a simple and accurate microassay for cholesterol and cholesterol esters in serum prompted us to develop a method suitable for use with 0.05 ml. of serum, an amount easily obtainable by finger-tip puncture.

The procedure we have developed makes use of the sensitive iron reagent introduced by Zlatkis and associates,[2] as modified in this laboratory,[3] and requires the minimal equipment and glassware available in all laboratories.

METHODS

A. Reagents.—

Iron reagent: Prepared as described by Rosenthal, Buscaglia, and Pfluke.[3]

Digitonin solution (0.5 per cent): Place 0.5 Gm. digitonin (Hofman LaRoche Co.) in a graduated cylinder. Add 53 ml. ethyl alcohol (95 per cent), 1 ml. of 10 per cent acetic acid, and dilute to 100 ml. with water. Mix to dissolve. Filter immediately before use.

Acetone-alcohol: Mix 1 part acetone with 1 part ethyl alcohol (95 per cent).

Glacial acetic acid: Analytical reagent grade.

Cholesterol standard: Stock standard: Dissolve 111 mg. dry cholesteryl acetate (Eastman Kodak Co.) in 100 ml. acetone-alcohol solution (1 ml. = 1 mg. cholesterol).

Working standard: Dilute 5 ml. of stock standard to 100 ml. with acetone-alcohol solution.

B. Experimental Details.—The absorption spectrum of the purple condensation product of pure cholesterol with ferric iron in acid solution, as prepared in the analytical procedure, exhibits a complex spectrum with a predominant major peak absorbing at 560 mμ which is useful for colorimetric analysis (Fig. 1, Curve A). However, shoulders with considerable absorptivity occur between 370 to 430 mμ and 430 to 519 mμ. The analogous spectrum of an equivalent amount of cholesterol digitonide exhibits an even more complex distribution of absorption maxima at 384, 426, 476, and 560 mμ (Fig. 1, Curve B). The increased absorbance at wave lengths below 500 mμ with cholesterol digitonide is due to the reaction of digitonin itself which reacts with the iron reagent to yield yellow colors (Fig. 1, Curve C). However, digitonin contributes minimal absorbance in the vicinity of 560 mμ. The lack of interference of digitonin at this wave length makes the determination of cholesterol esters in the presence of digitonin possible. The inclusion of digitonin makes the final reaction product appear orange red rather than purple due to the masking effect of the yellow compounds absorbing below 500 mμ.

The formation of the colored reaction product of the cholesterol iron complex in the presence of constant amounts of digitonin follows Beer's law in a fashion similar to that for free cholesterol or its esters as adequately reported by other investigators.[2]

C. Procedure.—With the above information and the conditions of the reaction previously defined,[3] it was possible to develop an accurate micromethod for analysis of cholesterol as described below. Cutaneous blood, obtained by puncturing the finger, ear lobe, or heel, is collected in heparinized capillary tubes as described by Natelson and associates.[4]

1. Extraction: To a graduated centrifuge tube containing about 2 ml. acetone-alcohol solution, add 0.05 ml. serum and mix. Place tube in a boiling water bath and bring

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solvent just to boiling. Remove tubes, cool to room temperature and adjust volume to 2.5 ml. with acetone-alcohol. Stopper, mix, centrifuge tubes quickly, and decant supernatant extract for analysis.

2. Fractionation of cholesterol and esters: Place 2 ml. of extract in a glass-stoppered centrifuge tube. Add 1 ml. filtered digitonin solution and mix tube by tapping. For the estimation of total cholesterol, immediately remove 1 ml. of mixture and place in a 50 ml. Erlenmeyer flask. Evaporate solvent on steam bath (85° C.) until residue is dry. A stream of warm air or nitrogen may facilitate drying.

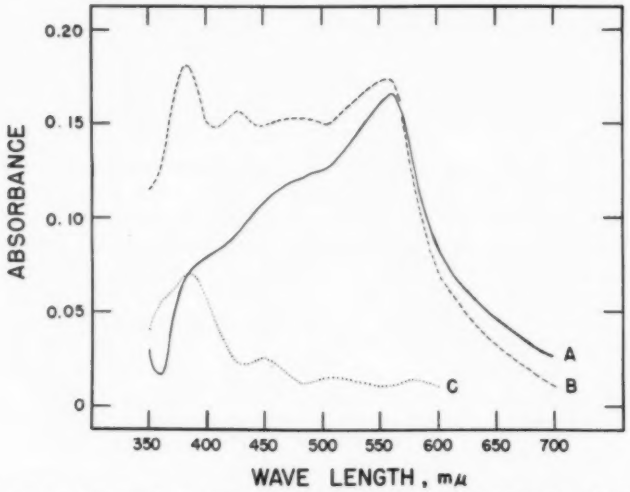

Fig. 1.—Absorption spectra of reaction product for cholesterol, cholesterol digitonide, and digitonin with the iron reagent. The spectra were obtained with a Beckman DU spectrophotometer and a 1 cm. light path. *Curve A*, cholesterol, 8 μg/ml. final reaction mixture. *Curve B*, cholesterol digitonide, 33 μg/ml. final reaction mixture. *Curve C*, digitonin, 50 μg/ml. final reaction mixture.

The remaining digitonin-extract mixture is tightly stoppered to prevent evaporation and allowed to stand at least 4 hours or overnight to precipitate the free cholesterol. After complete precipitation, centrifuge the tubes quickly and decant supernatant solution into a test tube. Pipette 1 ml. of supernatant into a 50 ml. Erlenmeyer flask and evaporate solvent on steam bath until residue is dry.

For routine analysis, a standard and reagent blank are prepared with 2 ml. of working standard and 2 ml. of acetone-alcohol, respectively, which are carried through the fractionation procedure described above.

3. Colorization: To the dry residues add 2.5 ml. glacial acetic acid and mix to dissolve residue. Add 2 ml. iron reagent to form a layer beneath acetic acid. Mix gently but thoroughly by rotation of flask, and allow the color to develop at room temperature for 30 minutes. Determine the absorbance of standard and unknown samples at 560 mμ with the instrument adjusted to 0 absorbance against the reagent blank. We have found the Coleman Junior spectrophotometer with 12 × 75 mm. cuvettes or the Sheard Sanford colorimeter to be satisfactory.

4. Calculations: Although cholesteryl acetate is used as the standard, all calculations are in terms of cholesterol. The equation is used for both total and esterified cholesterol.

$$(1)\ \frac{(C)\ (Au)}{As} \times 2{,}500 = \text{mg./100 ml.}$$

where C = standard cholesterol (0.1 mg./tube); Au = absorbance of unknown; As = absorbance of standard.

$$(2)\ \frac{\text{Ester cholesterol}}{\text{Total cholesterol}} \times 100 = \text{per cent esterified cholesterol.}$$

RESULTS

The validity of the cholesterol microanalytical procedure proposed in this report was determined by comparing the method with an acceptable existing macro procedure. The data were obtained on unselected serum samples from the routine laboratory. Total cholesterol was determined on acetone-alcohol extracts of serum by the method of Zak and associates,[5] as modified in this laboratory.[3] The fractionation of cholesterol and cholesterol esters was accomplished essentially in accord with the procedure of Sperry and Webb.[6] Final estimations of cholesterol and esters were performed with the iron reagent of Rosenthal and associates.[3] All determinations were performed in duplicate.

As shown in Table I, the micro procedure compares favorably with the macro procedure. A statistical analysis of the comparative data by means of

TABLE I. COMPARISON BETWEEN THE MICRO- AND MACROANALYTICAL PROCEDURE FOR CHOLESTEROL ANALYSIS

SAMPLE	NO. OF DETERMINATIONS	MACROANALYSIS	MICROANALYSIS	"t"
Total cholesterol (mg./100 ml.)	17	200 ± 13*	231 ± 18*	0.81
Range		120 – 368	135 – 402	
Esters, per cent of total	13	68.9 ± 2.28	67.7 ± 1.55	0.44
Range		50 – 78	60 – 76	

*Standard error.

Student's "t" test indicates no significant difference between the mean values of the macro and micro procedures. Recovery studies for total cholesterol were performed on serum samples to which cholesteryl acetate was added. The samples were then extracted and analyzed in duplicate by the proposed procedure. The recovery of added cholesteryl acetate, shown in Table II, is satisfactory.

TABLE II. RECOVERY OF ADDED CHOLESTERYL ACETATE AND CHOLESTEROL BY THE MICROANALYTICAL PROCEDURE

ANALYSIS	NO. OF DETERMINATIONS	ADDED MG./100 ML.		RECOVERED (PER CENT)
Total cholesterol	7	Cholesteryl	50	101 ± 4.9*
	7	acetate	100	99 ± 3.4
	7		200	102 ± 2.2
Ester cholesterol	5	Cholesterol	50	–0.0 ± 6.8
	6		100	–0.7 ± 3.6
	6		200	–0.8 ± 0.8
Ester cholesterol	6	Cholesteryl	50	102 ± 1.9
	6	acetate	100	102 ± 1.2
	6		200	101 ± 1.0

*Standard deviation.

The micro procedure measures the amount of esterified cholesterol remaining in the supernatant following precipitation of free cholesterol with digitonin. In order to test the completeness of digitonin precipitation, recovery studies were

performed by adding cholesterol to a group of sera and determining the amount of added cholesterol remaining in the supernatant solution. On analysis, it was found that precipitation of the added cholesterol was complete and none was recovered in the supernatant as shown in Table II. Further recovery studies were performed by adding cholesteryl acetate to sera and determining the quantity of added ester remaining in the supernatant. It is evident (Table II) that added cholesteryl acetate was completely recovered in the micro procedure.

DISCUSSION

The proposed cholesterol micro procedure derives its advantage from the ability to determine both total and ester cholesterol directly on the same aliquot of extract derived from 0.05 ml. of serum. Since the iron reagent yields the same color equivalent with either free or esterified cholesterol,[2, 3] preliminary saponification of serum cholesterol esters is not necessary. The micro procedure described in this report is especially suitable where large numbers of sera must be analyzed routinely since the direct determination of cholesterol esters remaining in solution following digitonin precipitation of "free" cholesterol eliminates the lengthy and troublesome process of washing cholesterol digitonide precipitates. It is evident from the recovery data that the inclusion of digitonin does not interfere with the procedure.

Although it is not the purpose of this report to discuss the precipitation of cholesterol with digitonin, it is pertinent to note that the digitonin step is the least precise aspect of cholesterol analysis. It was observed during this study that serum extracts do not show any visible precipitation for at least 3 to 5 minutes after the addition of digitonin solutions. In contrast to serum extracts, solutions of pure cholesterol begin to precipitate within a minute following digitonin addition. Cholesteryl acetate was selected for the standard in order to avoid the possibility of improper sampling of the standard solution prior to the removal of an aliquot for total cholesterol estimation. In order to avoid digitonin separation, Wycoff and Parsons[1] have recently developed an elegant procedure for separating cholesterol and its esters by chromatographic separation of serum extracts on silica gel columns. However, the preparation of columns for routine analyses may be tedious, time consuming and not suitable to the analysis of many samples.

SUMMARY

A micromethod for estimation of cholesterol and its esters in 0.05 ml. of serum is reported. The method compares favorably with existing macro procedures.

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AN IMPROVED TECHNIQUE FOR ASSAY OF $C^{14}O_2$ IN EXPIRED AIR USING THE LIQUID SCINTILLATION COUNTER

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A MAJOR problem in clinical and biologic investigations involving the use of carbon[14]-labeled materials in vivo is the assay of the isotope in the expired air. The necessary restriction of dosage to a few microcuries, especially in normal control subjects, often requires measurement of $C^{14}O_2$ of very low specific activity. The preparation of barium carbonate samples is cumbersome and involves the limiting factor of self-absorption when assays are made by conventional flow-gas counting. This has been overcome under certain conditions by the use of gas proportional counters and, more recently, by ion-chamber counting. An elegant method using the latter technique has been reported by Baker, Talbert, and Marcus[1] permitting continuous assay of expired $C^{14}O_2$. However, these methods usually require either careful precautions to purify the gas sample or instruments of very limited availability. The recent development of commercially available liquid scintillation spectrometers has afforded a greatly widened opportunity for assay of the weaker beta emitters at low activity levels. Since the phosphors now in use require the use of organic solvents, the counting of appreciable quantities of carbon dioxide was not possible until the ingenious use of a quaternary amine for dissolving carbonate in toluene-methanol mixtures was reported by Passmann, Radin, and Cooper.[2] These workers trapped carbon dioxide as sodium or barium carbonate, then transferred it to the organic base by liberation with acid in a diffusion vessel. The primary trapping of carbon dioxide in aqueous base from large (1 to 2 liters) samples of expired air and subsequent transfer to small volumes of amine solution is impractical, however, and it has been found feasible to eliminate this step. A system is described which utilizes direct collection of carbon dioxide in the organic base, permitting the rapid determination of total and specific activities of expired $C^{14}O_2$ with a minimum of time and manipulation. The method may be adapted to analysis of air samples separately collected from humans or large animals, or for continuous direct collection of carbon dioxide expired by smaller animals such as the rat.

MATERIALS AND METHODS

Chemicals.—Hyamine 10-X (methylbenzethonium chloride) was obtained from Rohm and Haas Co., Philadelphia, Pa. The amine-chloride was recrystallized 4 times from toluene and converted to the base as described by Passmann and associates.[2] A stock solution (1.0

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M) was prepared in methanol and subsequently diluted for use. 2,5-Diphenyloxazole (DPO) was obtained from Pilot Chemicals, Waltham, Mass. Reagent grade methanol and toluene were used without further purification.

Counter.—The liquid scintillation counter used was a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., La Grange, Ill.).

Gas Analyses.—Carbon dioxide in standard gas mixtures was determined in a Scholander gas analyzer which had been previously calibrated with a Haldane burette.

Apparatus.—The analytic train used for trapping the carbon dioxide from accurately measured volumes of expired air is shown in Fig. 1. It consists of the following units, connected serially: A. Douglas bag, made of nylon and rubber, containing the expired air.

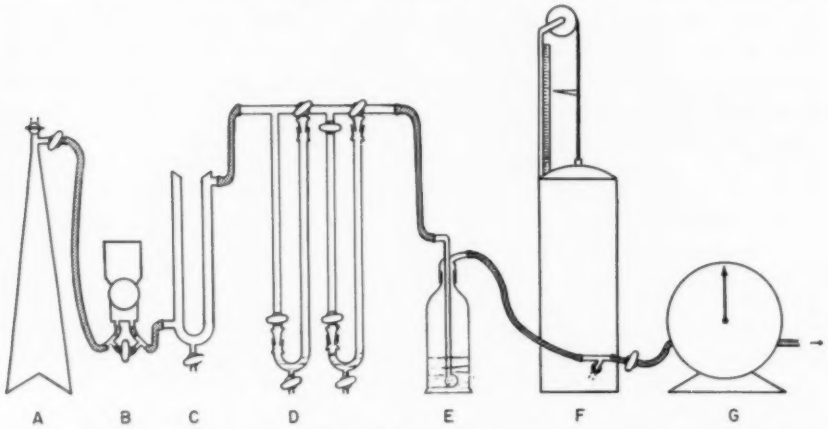

Fig. 1.—Schematic drawing of the system for $C^{14}O_2$ analysis. *A*, Douglas bag; *B*, pump; *C*, water trap; *D*, CO_2 trapping manifold; *E*, $Ba(OH)_2$ safety trap; *F*, spirometer; *G*, gas meter.

B. Diaphragm activated Dynapump* which is fitted with a simple throttle as indicated. C. Water trap (Dry Ice–acetone). D. Trapping manifold, consisting principally of two U-tubes, 45 cm. high, made from glass tubing of 10 mm. I.D. and fitted with ground-glass joints and outlets with stopcocks. The U-tubes are readily detachable from the remaining manifold. The upper horizontal limb of the manifold is fitted with two three-way stopcocks to permit the use of both U-tubes in series, or the by-passing of one or both. The proximal arm of each U-tube is foreshortened to minimize the column of "dead" air to be pushed through the trapping solution ahead of the actual air to be analyzed. E. Gas bottle containing $Ba(OH)_2$ solution, utilized as an indicator of trapping efficiency. F. Spirometer (obtained from the Sanborn Co., Cambridge, Mass.). G. Gas meter (made by the American Meter Co., Albany, N. Y.).

Method.—The analytic procedure consists in the rapid passage of a measured volume of dry air through a solution of Hyamine in methanol. All of the carbon dioxide is removed and the amount present determined by titration with dilute acid. A separate aliquot of the trapping solution is added to a toluene-DPO solution for assay of radioactivity in the scintillation counter.

When analysis is made of samples of expired air first collected in a container such as a Douglas bag, the procedure is as follows. Ten milliliters of a 0.5 M methanolic solution of Hyamine are placed in one of the U-tubes, which is then placed in position. The stopcocks isolating it from the rest of the manifold are closed. The bag is connected to the

*Obtained from Fisher Scientific, Pittsburgh, Pa.

pump and about 3 liters are flushed through the system exclusive of the U-tube and the spirometer. The spirometer is then connected, the stopcocks on the manifold are adjusted to direct the flow through the amine solution in the U-tube, and the desired sample is pumped through into the spirometer. A rate of flow up to 800 ml. per minute may be used with 100 per cent trapping efficiency. A convenient sample volume is about 2,000 ml. This volume of air expired by a quietly breathing human contains roughly 3 mM CO_2 or 40 per cent less than the capacity of the 0.5 M amine-base solution. After the pump is stopped, the U-tube is sealed by readjustment of the stopcocks and the spirometer volume recorded. A duplicate sample may then be similarly collected in the alternate U-tube, and finally the remaining contents of the bag drawn through the meter for calculation of the total volume, and thus the total radioactivity expired. The U-tubes are removed and the trapping solution drained into a graduated vessel, with a 0.5 ml. methanol rinse. To a 1 ml. aliquot, 5 ml. of 3 per cent aqueous $BaCO_3$ solution, and one drop of phenolphthalein are added and the cloudy solution is titrated with 0.1 HCl until the pink color disappears. For the most accurate blank it has been found best to reproduce the exposure of the amine to room air involved in the analyses by placing 10 ml. of the trapping solution for a few minutes in a U-tube closed from the rest of the manifold. The solution is then handled identically to the actual samples. The milliequivalents of amine base (R_3OH) replaced by carbonate are calculated by difference between blank and sample titrations. Comparison with CO_2 analyses obtained by the Scholander technique indicates the displacement of base by carbonate to be stoichiometric on a mol:mol basis. Two milliliter aliquots of the trapping solution are also added to the 20 ml. scintillation counter vials containing 15 ml. of 400 mg. per cent solution of DPO in toluene. The radioactivity in the vial is counted after chilling to −10° C. The specific activity in c.p.m./mM CO_2 is then readily calculable. Exact determination of total expired carbon^{14} in the air collected requires correction of the sample volume for the partial volume of the CO_2 removed. This may be done simply by adding to the volume recorded on the spirometer the product of: mM CO_2 in the sample, as obtained by titration, times the volume occupied by 1 mM of gas. (This volume is 24.2 ml. at 24° C. and 760 mm.; no correction for water vapor is required, since the dry air is re-equilibrated with this gas in the spirometer bell, at the time the volume is measured.)

In experiments involving small animals such as the rat, carbon dioxide may be collected directly for long continuous periods or samples taken intermittently. The animal is placed in a suitable metabolism chamber, ideally a glass tube with minimal dead space. The chamber is connected proximally to a source of CO_2-free air and distally to the pump (Fig. 1, *B*). The effluent air is then directed through alternate U-tubes, allowing time for rinsing and refill. Ten milliliters of 1 M amine base in methanol will suffice to capture all the CO_2 expired by a 250 gram rat in approximately 45 minutes, using a flow rate of about 600 ml. per minute. Simpler trapping systems may also be employed for these direct collections such as the substitution of gas bottles for the manifold. If larger volumes of trapping solution are desired, the methanolic amine solution may be diluted with toluene. This reduces the quenching effect due to methanol and permits larger aliquots for counting. Such dilute solutions are then titrated with very dilute alkali using a microburette.

Counting.—The addition of the methanolic amine solution to the toluene-DPO mixture reduces the efficiency of counting. As described by Passmann, Radin, and Copper,[2] quenching has been found to be due to both the presence of methanol and the Hyamine. In the aliquots used, the presence or absence of carbonate (final concentration 0.0 to 0.8 mM CO_2) does not affect quenching. With each series of analyses, the quenching effect is determined by addition of a standard solution of toluene-soluble, carbon^{14}-containing material to vials containing the amounts of methanol-amine solutions employed in assaying the CO_2 samples. No uniform correction factor can be employed for all batches of the amine, since in preparation of the amine base, small variable amounts of amine-chloride remain, unconverted to base. The total amine concentration is thus not obtainable by titration.

The absolute detection efficiency for C^{14} in the carbonate samples prepared in this manner was estimated by collection of labeled gas liberated from a Bureau of Standards solution of $NaHC^{14}O_3$. The diffusion technique of Passmann and associates[2] was used and the

$C^{14}O_2$ was trapped in 0.5 M amine solution in methanol. Assuming liberation and recovery of theoretical amounts of the CO_2, the efficiency obtained was approximately 29.5 and 23.4 per cent in vials containing 1 and 2 ml., respectively, of 0.5 M amine solution in methanol diluted to 17 ml. with toluene-DPO solution. The detection efficiency for palmitic acid-C^{14} in toluene-DPO alone was 54 per cent when identical photomultiplier voltage and pulse height discrimination were employed.

RESULTS

The reproducibility of sampling from a standard CO_2-air mixture is within an error of ± 0.5 per cent as indicated by the experiment shown in Table I. Also indicated in this table is a comparison of analyses of the same gas mixture by the Scholander technique and by the present method. In the latter method the CO_2 content was calculated from the titrations by assumption of a mol:mol ratio of carbonate to amine base. On this basis, the agreement obtained was excellent. Repeated experiments have been made in which gas mixtures containing approximately 5 per cent of both labeled and nonlabeled CO_2 have been passed serially through amine solution contained in both U-tubes. At rates of flow up to 800 ml. per min., all of the carbon dioxide has always been recovered in the first tube.

TABLE I. COMPARISON OF ANALYSES OF CO_2 CONTENT OF STANDARD CO_2-AIR MIXTURE BY THE PRESENT TECHNIQUE WITH THE SCHOLANDER GAS ANALYSIS METHOD

	mM CO_2 PER LITER
Amine	1.99
Amine	1.97
Amine	1.97
Amine	1.99
Scholander	1.98

DISCUSSION

The present technique expands the versatility of liquid scintillation counting to include the rapid analysis of $C^{14}O_2$ in large samples of expired air. The elimination of the step involving primary isolation of carbonate in aqueous base[2] greatly reduces the time and operations required for analysis with no sacrifice in sensitivity or reproducibility. Six to eight analyses per hour can easily be completed, exclusive of the time required for counting. Accurate measurement of the specific activity and total radioactivity in the expired $C^{14}O_2$ may be obtained from a two liter sample of air from a human subject expiring less than 1×10^{-6} mc per minute. We have successfully employed this method in the determination of the radioactivity expired by humans for many hours after the intravenous administration of long chain fatty acids in doses of 0.001 to 0.005 mc.

Where determination of specific activity only will suffice, equipment much simpler in design may be utilized. A simple U-tube may be substituted for the manifold (Fig. 1, *D*). Less accurately measured volumes of air may be passed through the amine using devices less costly than the pump and spirometer. For example, the displacement of water from a large calibrated jar may be used for

this purpose. The removal of water vapor has been found to be essential, however, to prevent cloudiness in the amine-methanol-toluene mixture. No significant loss of CO_2 through solution in the water vapor sequestered in the Dry-Ice–acetone trap has been detected.

SUMMARY

A rapid and efficient system is described which facilitates the analysis of $C^{14}O_2$ in large samples of expired air. The method employs a modification of the method of Passmann, Radin, and Cooper,[2] using methylbenzethonium chloride (Hyamine-10-X) for solubilization of carbonate in toluene. The initial step involving trapping in aqueous base has been eliminated. Radioactivity is measured in a liquid scintillation spectrometer. The total $C^{14}O_2$ radioactivity and specific activity in air from a human expiring less than 1×10^{-6} mc. per minute may be accurately measured with a minimum of time and manipulation. The direct trapping and analysis of carbon dioxide expired by small animals is also described.

The authors wish to express their appreciation to Mr. Freeman Bradley for Scholander gas analyses and to Dr. Frank Eisenberg for several helpful suggestions.

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OBJECTIVE INTERPRETATION OF PRECIPITATE PATTERNS IN AGAR DIFFUSION PRECIPITIN PLATES

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SIMPLE diffusion techniques, of which the Oudin tube[1] is the prototype, lend themselves to accurate instrumental measurements of the course of diffusion as indicated by the progressive deposition of specific precipitate. Double diffusion plates of the Ouchterlony type,[2] on the other hand, offer several advantages with respect to the identification of particular precipitin systems in complex mixtures, but have largely been interpreted on the basis of subjective judgment of the forms taken by the precipitates deposited. The triangle plate[3] combines the advantages of the two techniques, retaining the "built-in" control arrangement typical of the Ouchterlony plate but permitting interpretations to be based on accurate measurements of the geometry of the precipitate pattern.

The plate illustrated in Fig. 1, *A* was devised by William Kenny, of Skill Alliance, and offers several advantages over the earlier models previously described.[3] These plates, which may be purchased from A. S. Aloe, have recessed bottoms which are not scratched by contact with the desk, and do not have the notches previously used to guide the knife in dividing the agar. Instead, the special knife illustrated is guided by flanges fitting over the outside of the plate. Three milliliters of 1 per cent clarified agar containing buffered saline and preservative will harden in such a plate to form an equilateral triangle with 50 mm. sides. The knife is then pressed into the agar, and the corner quarter remaining exposed is removed, as shown (Fig. 1, *B*), by means of a spatula. Any remaining agar or fluid may be removed with tissue.

The knife is then withdrawn, and the depot is refilled (Fig. 1, *C*) with 0.5 ml. of 1 per cent agar containing the appropriate solution of antigen or antibody. When this has hardened, the process is repeated on each of the two remaining corners.

The resulting arrangement, like the Ouchterlony plate, presents three depots separated from each other by an intervening arena of saline agar into which all reactants must diffuse. The unknown may be placed in one depot and an indicator solution—antibody, where the unknown is an antigen, or vice versa—in a second. It the identity of the unknown is in question, a reference reactant known to form precipitate with the indicator may be placed in the third depot, or, if desired, a second specimen of the unknown may be examined under conditions identical to the first, as shown in Fig. 2.

RESULTS

Zone Formation in the Triangle Plate.—Precipitate in the agar of a diffusion-precipitin plate or tube marks the location of equivalent concentrations of antigen and antibody established at the point observed in the course of

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diffusion. Diffusion-precipitin analysis is therefore a matter of estimating the advance of molecules falling into the category dictated by the ability to form specific precipitate, one which may or may not correspond to the categories into which the physical chemist would divide the components of the mixture subjected to assay. Thus, a bacterial culture filtrate may contain toxin molecules which qualify as antigen and polymers of those molecules which will be considered the same antigen by the test employed, even though they may differ markedly in those properties which determine the diffusion coefficient.

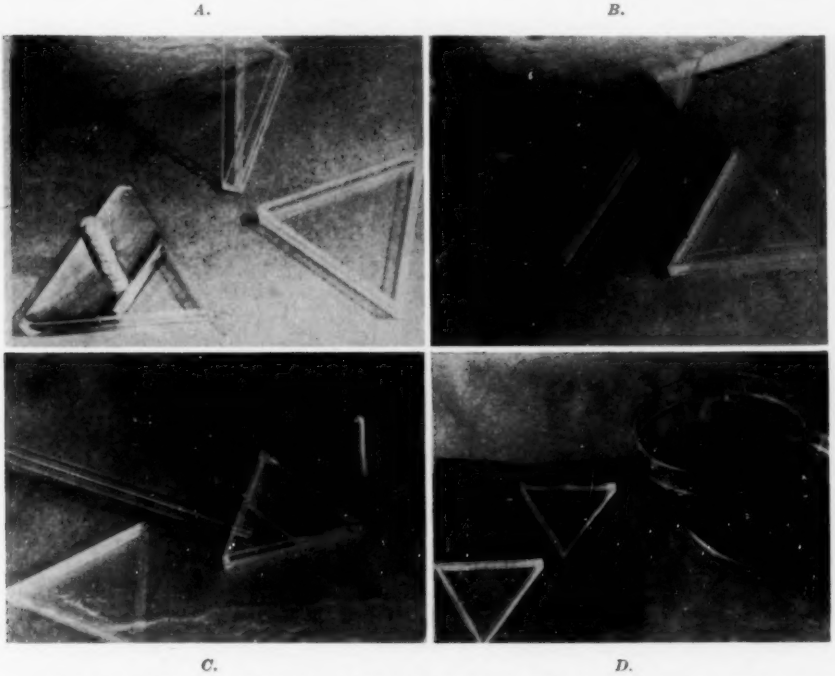

Fig. 1.—Preparation of triangle plate. *A*, Plate, showing recessed bottom and use of knife. *B*, Preparation of reactant depot. *C*, Filling depot. *D*, Plates with typical zones, after 8 hours' diffusion. Petri dish used to provide atmosphere saturated with water vapor during incubation is shown on right.

In effect, we neglect all the molecules which do not possess the necessary label of immunologic specificity, and consider the behavior of all those that do, however physically heterogeneous the labeled population may be. Furthermore, we make our observations, not on the behavior of the population as a whole, but on one particular definitive concentration within the gradient of concentrations established by diffusion. In assays such as those performed by Becker and associates,[4] by Rubinstein,[5] and by Glenn[6] using the advance of the zone away from the antigen depot in an Oudin tube as the measurable

attribute, the definitive concentration will vary from experiment to experiment being equivalent to the concentration of indicator employed. A slow zone, resulting from the use of a high concentration of antibody in the agar column, will nonetheless be reflecting the same things as will a more rapid zone moving through a more rarefied atmosphere of antibody: namely, those properties of the antigen solution which control the expansion of the gradient as a whole. These are, first, the number of identifiable molecules seeking escape from the depot and, second, the average speed with which the individual molecule advances. The definitive concentration is a particular number of specific determinant groups which will be a given distance from the source in a short time if the average labeled molecule is small and symmetrical, or which will arrive at the same point after a longer period if the molecules are such as to move more deliberately. It is convenient to think of the gradient as rank upon rank of isobars, representing the momentary location of a particular number of determinant groups, each more or less parallel to the source of diffusion and moving away from it at a characteristic rate determined by concentration and diffusion coefficient—if we may use that term in dealing with a nonuniform molecular population. We have suggested that the composite property of the unknown which is thus measured be referred to as "diffusivity."[7]

In the triangle plate, the isobar of antibody representing that which will give the minimal amount of precipitate visible in the agar containing it will meet the equivalent concentration of antigen at a point just within the apex of the arena separating the two depots as soon as diffusion begins. The progress of these two isobars of threshold concentrations, which will not vary from one experiment to the next with differences in depot concentration as do the definitive isobars in simple diffusion arrangements, will determine the path along which precipitate is initially deposited.

The location of these threshold isobars at a given moment is suggested by the diagram in Fig. 2. If, as with the unknown in concentration C_x, the advance of the definitive isobar of antigen balances that of the advance of the equivalent concentration of the standard antibody, the path of the point of intersection of the two isobars will bisect the angle between the depots. This will be the path of zone growth along which the initial deposit of precipitate occurs and will, in fact, correspond to the path of intersection of subsequent higher ranking equivalent concentrations of antigen and antibody. All the precipitate will be deposited in the ribbonlike confines of this path.

Given a second sample of the antigen containing fewer molecules, the gradient will expand less rapidly, and the definitive isobar will advance more slowly, as suggested for zone Y in Fig. 2. The path of zone growth will still be a straight one, but the angle between the path and the face of the antigen depot will be more acute than that with the face of the antibody depot. Furthermore, higher concentration isobars will intersect on paths each a little nearer the antigen source than the previous one, so that new precipitate is constantly deposited antigen-ward of the old. What is observed is migration of

the zone, as an Oudin zone migrates, along the wall of the vessel into the antigen depot while the zone grows. The result experimentally is a triangular deposit of precipitate as shown.

Where the diffusivity of the antigen is greater than that of the antibody, as with zone Z in Fig. 2, the same phenomenon takes place in the opposite direction. At the same time, precipitate once deposited is bathed in an ever-increasing excess of the antigen, and will be more or less redissolved according to the properties of the particular precipitin system involved. The zone may be quite narrow, but will migrate along the wall into the antibody depot while its tip describes a path closer to the antibody source than to the antigen source.

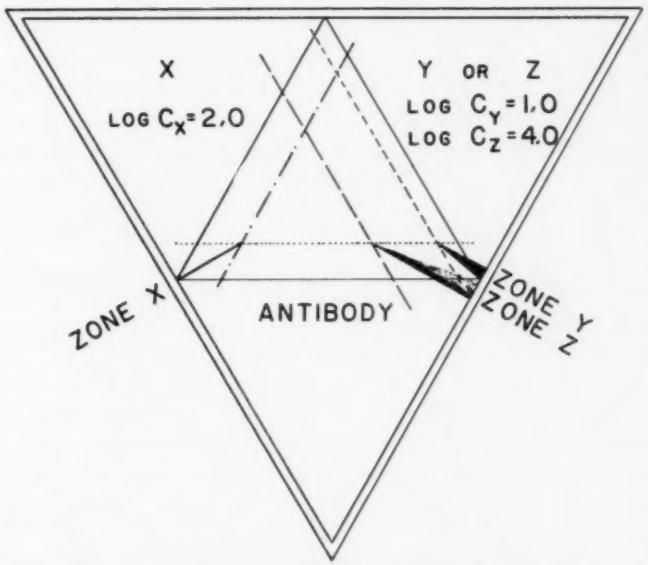

Fig. 2.—Normal zone forms. Broken lines represent hypothetical location of points of distribution of threshold concentrations of antigen and antibody at a given moment.

We have pointed out[8] that the direction and extent of zone migration along the wall of the plate may be used, at least subjectively, as an index to the diffusivity of an unknown as it is in the Oudin tube. Consideration of the diagram, however, will show that this migration is rather small as compared to zone growth, and will in fact be maximal when the precipitate is deposited in a relatively large volume of agar making it difficult to observe. The same factors which control the migration, however, control the direction of zone growth, which may be measured even in the absence of migration. The path of zone growth has been found experimentally to be straight for a reasonably long period of time, and may be established confidently by projecting the line defined by the precipitate formed in the early hours of diffusion. In fact, it

was this linearity which led us to modify the original Ouchterlony arrangement, in which the path of zone growth is curvilinear to a variable degree,[9] according to the relative diffusivities of the antigen and antibody solutions employed. We have chosen the direction of zone growth as the criterion of assay, but call attention to the fact that the properties analyzed are the same as those reflected by measurements of zone migration.

The Diffusivity Ratio.—The direction taken by the path of zone growth is controlled by the relative diffusivity of the unknown with respect to the diffusivity of the indicator. The distance, A, in Fig. 3, is the distance which will be traversed by the definitive threshold concentration of the unknown while the equivalent concentration of the indicator traverses the distance, B. The diffusivity ratio of the unknown, A/B, is the distance the gradient of the unknown will expand while the gradient from a depot containing a standard indicator solution expands one millimeter.

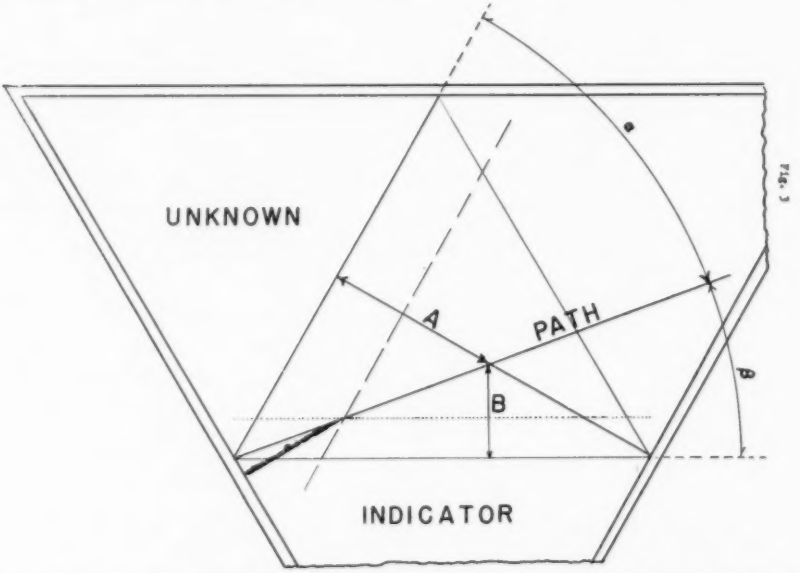

Fig. 3.—Geometry of zone growth. Broken lines represent distribution of threshold concentrations of reactants at given moment. Unknown represents depot containing antigen or antibody to be assayed. Indicator shows depot containing appropriate reactant capable of forming specific precipitate with unknown. Path illustrates line of deposition of initial precipitates.

Jennings and Malone[7] describe a method of arriving at the diffusivity ratio by linear measurements along a filament mounted on the observation stage, on which the plate is placed with the face of the unknown depot at right angles to the filament. The path of zone growth is defined by a second filament adjusted to register with the apex of the arena and the tip of the zone. Advantage is taken of the fact that the hypotenuse of the triangle whose base is B is twice the length of the base: A and B may both be measured by a cathetometer traveling along the reference filament.

However, while the method is feasible and will give accuracy sufficient for many practical purposes, the location of the three points necesary in measuring A and B is technically difficult. Studies are in progress aimed at the reduction of experimental error.

It will be noted that the sine of the angle alpha, between the unknown depot and the path of zone growth, divided by the sine of the angle remaining to the indicator depot is also A/B. Since a filament had to be aligned with the path of zone growth, an attempt was made to mount this in a protractor, permitting its alignment with the face of the unknown depot, the path of zone growth and the face of the indicator depot in turn. This proved to be more convenient and more rapid, but no more accurate, probably because of the parallax occasioned by the rotation of the protractor filament in a plane different from that of the precipitates.

At present, the equipment has been modified by mounting the plate with the apex of the arena at the center of rotation of the stage of a petrographic microscope, which in turn is mounted over a suitable dark-field light source. A small telescope is used above this stage, with cross hairs also in register with the center of rotation of the stage. One of these hairs, whose image lies in the actual plane of the precipitates, serves as the index and the angular movement of the stage is noted on a scale graduated to 0.1 of a degree.

This is the most satisfactory arrangement tested as yet, but is probably one which can still be improved upon. The reproducibility of individual readings is not as great as desired, though quite satisfactory results are obtainable by the use of a sufficient number of replicate preparations for each unknown. In the examples cited here, plates were prepared in triplicate, and two series were examined, making six determinations for the establishment of each point.

Diffusivity Analysis.—Within the limits of experimental error, the relationships indicated by the equations shown in Table I have been found applicable with a wide variety of antigens and of antibodies.

TABLE I. DIFFUSIVITY RELATIONSHIPS

1. $R_{Cu} = A/B$ = sine alpha/sine beta
2. $R_{Ci} = B/A$ = sine beta/sine alpha = $1/R_{Cu}$
3. $R_{Cu} = K \log C_u + r_o$
4. $R_{Ci} = K' \log C_i + r_o'$

C_u = Concentration of unknown (antigen)
C_i = Concentration of indicator (antibody)
Alpha = angle between unknown depot and path of zone growth
Beta = angle between indicator depot and path of zone growth
A = perpendicular distance from unknown depot to point on path
B = perpendicular distance from indicator depot to same point
K = slope of regression line
r_o = intercept value = R_{Cu} where $C_u = 1$ unit/ml. ($\log C_u = 0$)

The first, describing the measurable aspects of the precipitate patterns which will serve as indices of the diffusivity ratio, has been discussed. The

second calls attention to the fact that the diffusivity of the indicator may be expressed as a factor of the diffusivity of the unknown quite as validly as the reverse.

The third equation states that a straight-line relationship exists between the diffusivity ratio, R, for a given concentration, C_u, of the unknown when compared to a particular indicator solution, and the logarithm of the concentration of the unknown. This may well be an oversimplification of the actual relationships, but if a curvature exists in the regression, it is too small to demonstrate without reducing the present experimental error still further. At extremely low concentrations of reactants, where precipitates are minimally dense and the experimental error is greatest, diffusivity ratios higher than the theoretical values for linearity have been observed with a fair amount of regularity. Over the greater part of the range of permissible antigen to antibody depot concentrations, giving diffusivity ratios both greater and less than one, however, no consistent departure from theoretical values has been observed.

The fourth equation indicates the effect of the concentration of the indicator employed on the rate of expansion of the indicator gradient. Since this varies with the logarithm of the indicator concentration, and since the diffusivity ratio of the unknown is related to the diffusivity of the indicator, it is obvious that the constants, K, expressing the slope of the regression line, and r_o, the intercept value, are applicable for but one concentration of indicator.

If we take a particular sample of antitoxin and reserve it for a laboratory standard indicator, we may establish the values of K and of r_o appropriate for that indicator by treating ratios observed with various known concentrations of toxin by the method of least squares. If now we take this same indicator and examine a series of dilutions of toxoid representing the same Lf values, we will again establish a linear relationship between the observed ratios and the concentration, but will find that the constants differ from those established with the toxin. We may assume that an Lf unit, whether toxin or toxoid, represents a certain definite number of specific determinant groups combining with antitoxin. The observed differences in diffusivity must be attributed to the physical differences between the molecules bearing these determinants, and the constant becomes, therefore, an objectively asertainable criterion of qualitative aspects of the unknown. How sensitive this test will prove to be remains to be seen, but two unknowns which show similar immunologic properties, but differ as to the K value in diffusion-precipitin studies, must be assumed to differ physically. Of course, this difference may be one of proportions of different types of molecules in a heterogeneous population, rather than purely a difference of kind. Apparently similar toxoid solutions have been found to show differences in K values, which are most probably attributable to this type of qualitative make-up.

The r_o value will be recognized as the diffusivity ratio calculated for an unknown solution containing one unit of the unknown per milliliter: that is to say, having a value of log C_u equal to 0. From equation 4, we may expect

that the reciprocals of the r_o values observed in a series of experiments with various dilutions of the indicator will be found to show a linear relationship to the logarithm of the indicator concentration. Experimentally, this is almost never true.

A typical experiment of this sort is summarized in Table II. Here the diffusivity ratios observed with 6 different concentrations of crystalline bovine serum albumin, when examined by 7 different dilutions of a stock solution of antialbumin, have been treated by the method of least squares to determine the constants shown for each of the several antibody dilutions. Note that the value of K increases regularly with decreasing indicator concentration as expected. The values of r_o and of $1/r_o$, however, vary in an apparently random fashion.

TABLE II. DIFFUSIVITY ASSAY OF BOVINE SERUM ALBUMIN

EXPERIMENTAL VALUES FOR 6 DILUTIONS OF ANTIGEN EXAMINED WITH FOLLOWING CONCENTRATIONS OF ANTIBODY AS INDICATOR							
Log C_i	1.778	1.602	1.477	1.176	1.125	0.875	0.648
K	0.454	0.519	0.629	0.733	0.888	1.310	2.128
r_o	1.036	1.054	1.090	1.209	1.143	1.275	1.189
$1/r_o$	0.965	0.948	0.917	0.827	0.875	0.784	0.841
$R_{10}\ \frac{1}{(K + r_o)}$	0.672	0.635	0.582	0.515	0.492	0.387	0.302
EXPERIMENTAL VALUES FOR 7 DILUTIONS OF ANTIBODY EXAMINED WITH FOLLOWING CONCENTRATIONS OF ANTIGEN (C_u) AS INDICATOR							
Log C_u	1.097	0.796	0.699	0.495	0.398	0.097	
K′	0.323	0.317	0.355	0.305	0.231	0.345	
r'_o	0.104	0.172	0.150	0.261	0.395	0.372	
R'_{10}	2.340	2.047	1.979	1.770	1.597	1.390	

Concentrations of antigens, mg./10 ml.
Concentrations of antibody in arbitrary units established by assigning a convenient value to stock solution from which dilutions were prepared.

This may indicate that the linear relationship postulated is incorrect, or it may simply reflect the inaccuracies of measurement at the present time. We are inclined to believe the latter to be the case, since when the intercept value is high, the slope appears to be low. When log C_u is 1.0, that is, when the concentration of the unknown is 10 units per milliliter, the diffusivity ratio will equal K plus r_o. The reciprocal of this value should be the diffusivity ratio of the particular indicator, as compared to 10 units of the antigen, and again we would expect a linear relationship to changes in log C_i. This is found to exist, suggesting that the variations in the intercept values are due to a few observations which are out of line, rather than to a consistent error in theory.

Since the reciprocals of the diffusivity ratios found for the antigen represent the ratios of the antibodies expressed in terms of the antigen as indicator, the same data may be treated to determine the values of K′, r'_o, and R'_{10}. The increase in K′ with decreasing concentrations of antigen, if it existed, was masked by the experimental error. The R'_{10} values, however, showed a consistent relationship, and we may judge that the actual changes in K′ with changing antigen concentration were quite small, as would be the

case if the antigen gradients all expanded more rapidly than the antibody gradients. This was borne out by the observed diffusivity ratios, those of the antigen to the antibody varying from 1.0 to 3.2 with an average of about 1.7, while those of the antibody to the antigen ranged downward from 1.0 with an average of 0.63.

The diffusivity ratios observed with actual tests of a series of unknowns examined by means of an indicator with a log C_i value of 1.0 agree excellently with those calculated from the over-all data as in this table. However, the calculated values have the advantage of being acted upon by the entire series of experiments, and the linearity of the observed regression is, perhaps, better evidence of the reality of the relationship as an hypothesis applicable to all cases. The results reported in Table II have therefore been plotted, as shown in Fig. 4, to permit visual comparison with the theoretical values represented by the line established by the method of least squares.

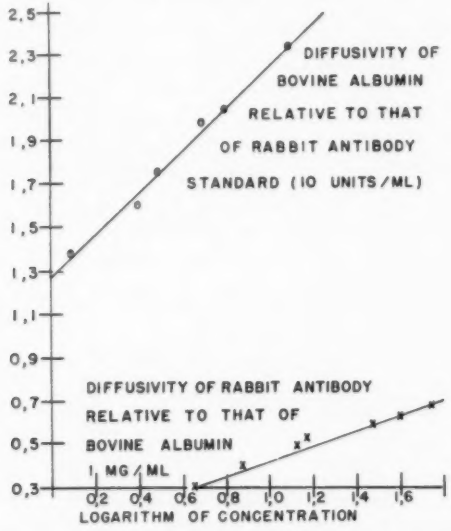

Fig. 4.—Linearity of regression. Circles: $1/(K' + r_o')$ established by method of least squares from experimental values found with 7 dilutions of antibody tested against indicated quantity of antigen. Crosses: $1/(K + r_o)$ values established from 6 experimental points with antigen dilutions for each of the indicated concentrations of antibody. Lines: theoretical values established from the data by method of least squares. Concentrations of antigen are expressed in mg./10 ml. Concentrations of antibody are expressed in arbitrary units based on assignment of convenient value (120 units/ml.) to concentrated solution of immune rabbit globulin.

Whether the regression line is established in this fashion, or in the simpler manner with a single standard indicator preparation, the possibility of performing quantitative assays on unknowns by extrapolation from such regression charts must be obvious. We have pointed out the danger of error in the event that the unknown represented a different proportion of various molecules satisfying the criterion for antigen or antibody than did the reference

material used in establishing the regression. Such error can be avoided by the simple expedient of examining each unknown in a series of 3 or 4 dilutions. The K value of the individual specimen may then be compared with that of the reference material and, if agreement is found, the quantitative assay may be considered reliable. That this is the case is attested to by the fact that these values shown in Fig. 4 represent accumulated data from two separate series of experiments, separated in time by some months, during which the reactants were stored, and that each of the series of experiments covered a period of about a week, during which an entire series of antigen dilutions was examined against one of the antibody dilutions each day. Thus, any of the points might be considered as an unknown being assayed in terms of the regression established by means of the others.

The work presented shows evidence of error which should be reduced. It also shows that a very useful relationship does exist which is capable of being measured accurately if the technique can be refined sufficiently. It is our hope that this demonstration will stimulate interest leading to the early solution of the remaining problems of technique and equipment. Attention is called to the potential advantages of the method: both qualitative and quantitative assays may be performed objectively; the mechanism permitting indentification of zones on the basis of coalescence or crossing, the plate being returned to the moist chamber for further incubation after readings are taken, is retained; zones begin to develop at once, and readings should be made before they have grown more than halfway to the center of the plate; therefore, assay requires from 4 to 24 hours, only. It should be possible to perform individual assays of each of several antibodies in single small specimens of serum, or of antigens in biologic fluids, within this length of time. Finally, the method offers a simple and reliable means of distinguishing between immunologically related, but physically diverse, reactants and may, therefore, prove useful in demonstrating that antigens, separated from their native mixtures, have not been altered physically in the course of the physical and chemical manipulations required.

SUMMARY

Depots of antigen and of antibody suspended in agar, presenting straight edges meeting at an angle in a plate of saline agar, produce zones of precipitate whose direction of growth with respect to the sources of diffusion reflect the relative rates of the expansion of the concentration gradients established by diffusion. Linear or angular measurements of the precipitate patterns permit objective estimation of the "diffusivity ratio" of the unknown antigen or antibody—the number of millimeters a definitive concentration will advance while the equivalent concentration of reactant from a standard solution of indicator antibody or antigen advances 1 millimeter.

The diffusivity ratio bears a straight-line relationship to the logarithm of the concentration of the unknown. With a given standard indicator, the

slope of the regression line reflects physical characteristics of the unknown, while the intercept value depends on physical characteristics and on the size of the unit chosen to express concentration.

Quantitative assays may be performed by extrapolation from a regression line established with known concentrations of the material to be assayed, provided that a series of concentrations of the unknown is shown to produce a regression with similar constants; failure to obtain similar values for the slope and intercept with a series of dilutions of the unknown may be taken to indicate the existence of physical differences between the unknown and the reference reactant used in establishing the standard regression formula.

The measurements may be made from projections of the precipitate patterns formed during the first 4 to 24 hours of diffusion. The method is, therefore, a rapid one and relatively free from errors introduced by uncontrolled variable factors such as temperature changes and inequalities in the agar which might influence the results during a more protracted incubation period.

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THE CONSTRUCTION OF A SENSITIVE ACCELEROMETER

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THE basic studies of Burger,[1] Talbot,[2-4] and Von Wittern[5] have shown that the acceleration of an ultralow frequency ballistocardiograph may yield accurate representation of cardiovascular force. These studies have resulted in a marked increase in research in this field of physiology. However, such investigations have been handicapped by the lack of accelerometers of suitable sensitivity and frequency response. The accelerometer should be capable of measuring forces of 5/10,000 g, and should have an undistorted frequency response range to at least 50 cycles per second. Such instruments are not readily available commercially.

An instrument apparently fulfilling these criteria was recently described by Elliott, Packard, and Kyrazis.[6] This accelerometer is an electrokinetic device based on the Ueda principle, and consists of alternating cellules of mercury and sulfuric acid in a capillary tube. When electrode tips are placed in the terminal cellules, an electrical signal can be recorded upon motion of the accelerometer. According to the description of Elliott and associates the instrument is easy to construct and has a linear frequency response to acceleration up to 2,000 c.p.s. It may be used with an ECG amplifier and recorder. Unfortunately, several laboratories[7, 8] have encountered great difficulty in the construction of this type of accelerometer with adequate output and stability characteristics, leading to abandonment of the technique in those laboratories. The present report describes refinements in technique of construction and considers possible factors responsible for variations encountered. Four major difficulties have been frequently found. First, a nonfunctioning accelerometer; second, inadequate voltage; third, marked instability of output with certain types of recorders; and fourth, sudden deterioration of an instrument, which formerly demonstrated good performance.

Variables Studied.—A number of variables were considered. It was found that commercial triple-distilled mercury used without further purification, and approximately half molar sulfuric acid solution, was adequate, i.e., further purification of the mercury or use of exactly one normal sulfuric acid did not influence the sensitivity or stability of the instruments built. Scrupulous washing of the capillary lumen with chromic acid and distilled water to remove greases failed to give any significant advantage. Kimball glass capillary tubing of 0.4 to 0.7 mm. was used in initial experiments, and in this range at least, capillary diameter did not appear to be a major factor in the extreme variations in output encountered.

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A meticulous examination of nonfunctioning accelerometers revealed one consistent defect. This was an air bubble interrupting the continuity of the mercury-sulfuric acid column. Examination of those accelerometers with inadequate output revealed either no air at all at the terminal ends, or interruption of the continuity of the mercury-sulfuric acid column by small bubbles of air.

The study of sealing techniques, as regard variations in output, gave the most significant information. It was noted during construction by the method of Elliott, Packard, and Kyrazis that insertion of plain fine copper wire into the terminal mercury pellets caused the introduction of a variable and uncontrollable amount of air, due to surface tension relationships between mercury, glass, and copper. The chance addition, therefore, of variable quantities of air at the ends of the accelerometers apparently allowed the interfaces of those in which the quantity added was relatively large to have a more free swing than those in which the quantity added was extremely minute. It was felt that this might be responsible for the differences encountered in voltage output between accelerometers. To check this hypothesis, accelerometers were constructed with exclusion of all air possible. This was accomplished by amalgamating the copper wire to provide complete wetting of the wire by mercury, and use of a cold polyethylene seal developed for the purpose, which provided visualization of the ends of the capillary tube during and after construction, so that accelerometers containing terminal air could be detected. It was found that when air was completely excluded from the capillary column, the output was under 0.05 millivolt to a force of 540,000 dynes applied to a low-frequency ballistocardiographic bed. The introduction of small quantities of air into the ends of these accelerometers was found to give markedly increased output. Using the same force input, outputs of 7 millivolts were easily obtained. Satisfactory results in the construction of mercury-sulfuric acid capillary accelerometers are, therefore, possible if the following procedure is used in which a small quantity of air is introduced at the capillary ends. This gives a voltage increase but does not give a detectable phase shift. However, if excessive quantities of air are added, a resonance artifact may be introduced.

Construction of Accelerometer.—The accelerometer is constructed as previously described,[6] using capillary tube 0.4 to 0.7 mm. in diameter. After filling with mercury and 0.5 N sulfuric acid, one end is closed with sealing wax. A fine coated copper contact wire, amalgamated over the terminal ¼ inch, is inserted at the opposite end for approximately ½ to ¾ inch. The enamel coating should be preserved about the proximal portion of the wire, to present a surface nonwettable by mercury. The displaced mercury is removed and the wire is then withdrawn slightly, until a suitable amount of air has been introduced (using a 0.5 mm. diameter tube and a No. 34 contact wire, a column length of air of approximately 1 mm. at each end of the capillary is adequate). Sealing wax is then applied, using a small soldering iron, to give an airtight seal. The other end of the tube is then nicked with an ampul opener and broken in such a way as to allow half an inch or more of mercury to remain in the end pellet. A small quantity of air is introduced in the same fashion as before, by manipulation of the contact wire, and sealing wax is then again applied. With the bubble of air resting on a section of unamalgamated wire, it was found that the surface tension relationships are such that the migration of the air bubble into the central portions of the tube was made more difficult and, therefore, sudden decrease in output was less frequently encountered. In some cases, it is necessary to readjust the amount of air trapped in the terminal ends of the tube by shaving off the sealing wax closure and manipulating the wire as before. The quantity of air may be increased until desired sensitivity is achieved by further insertion and withdrawal of the contact wire, or additional mercury added if sine wave resonance artifact is encountered. The assembled accelerometer is placed in a groove marked in a piece of balsa wood of suitable size, held in place with a small amount of collodion applied near the middle. The terminal wires are similarly attached and may then be connected to the amplifier-recorder, as previously described.

It has been found that in construction, ¼ to ½ inch pellets of mercury, and sulfuric acid may be used. Following complete assembly of the accelerometer on balsa wood, light

flicking of the instrument with the finger increases the number of interfaces by causing fragmentation of the pellets, a concomitant increase in voltage output results.

With careful construction, accelerometers made as described above have adequate output, but a certain percentage of these will leak mercury around the sealing wax, with subsequent introduction of excessive air. Furthermore, unless handled carefully in the horizontal plane, the air present at the terminal ends has a tendency to run along the wire until the continuity of the mercury column is broken. To avoid this difficulty, a design was

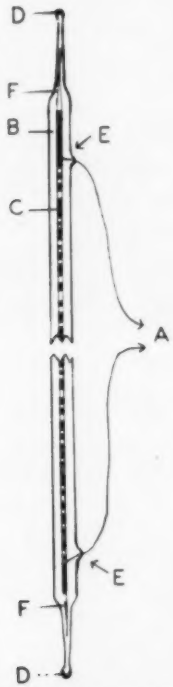

Fig. 1.—*A*, Output of lead wires. *B*, Glass capillary tubing. *C*, Mercury-sulfuric acid column. *D*, Sealed ends. *E*, Glass-to-metal seal. *F*, Trapped air.

developed (Fig. 1), consisting of fusion of two copper ribbons or platinum wires in the side of a thick-walled Pyrex capillary tube with an internal diameter of 0.5 to 0.6 mm. The contact wires protrude into the lumen of the tubing, approximately an inch or two from each end. The ends of the capillary tubing are then drawn down to fine threads. The tube is filled as described above, but prior to sealing ½ to 1 *cubic millimeter* of mercury is drained from one end. The capillary column is then adjusted by holding the tube in the horizontal plane to give equal air space at both ends, and a fine point flame is used to fuse the terminal capillary glass threads. With this construction, the air is more efficiently trapped, and the innate strength of the thick-walled capillary tubing makes the use of supporting materials unnecessary except as needed for sonic insulation and electrical shielding. As in the prior construction, the quantity of terminal air may be adjusted to give a higher voltage output by breaking off the tips of the end capillary threads and removing more mercury. As before, if too much air is removed a resonance artifact may appear. It has been noted also, that air bubbles smaller than the internal diameter of the capillary tube—no matter where placed—do not cause difficulty, but act to increase output.

DISCUSSION

At very low frequencies, an electrical phase shift may become apparent. Evidence has been adduced in this laboratory that the frequency response of these instruments may not be completely linear over the range concerned in ballistocardiographic measurements. By the use of suitable capacitance across the terminals ranging from 0.005 to 0.05 m.f.d. when used with direct writing recorders, an essentially linear response from one to 40 c.p.s. may be obtained. (Higher frequencies have not been tested.) It is, therefore, necessary to balance each accelerometer before use, and to check it at intervals thereafter. Such balancing may be accomplished by the use of a constant force shaker, or by testing against a known displacement or velocity pickup for phase shift and amplitude response.[9]

Accelerometers have been constructed in this laboratory ranging in length from 4 cm. to over 60 cm. and the number of mercury pellets have varied from 5 to more than 100. The output varies with the number of pellets, as has been previously described.[6] Satisfactory outputs have been obtained from instruments constructed with cellules varying from $\frac{1}{16}$ to $\frac{1}{4}$ inch.

SUMMARY

Voltage output of mercury-sulfuric acid capillary accelerometers is improved by small amounts of air introduced at either end of the capillary during sealing. Decrease in output or failure is usually due to interruption of the mercury-sulfuric acid column continuity by an air bubble. A method of increasing the number of mercury-sulfuric acid interfaces by impact is suggested. A modified glass sealed construction is described. Attention is drawn to the necessity of the individual testing of each accelerometer for phase and amplitude response to accelerations at the frequencies encountered in the phenomenon to be studied.

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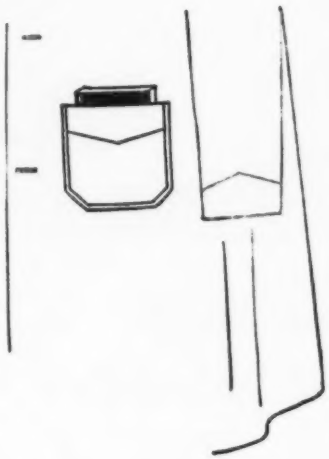

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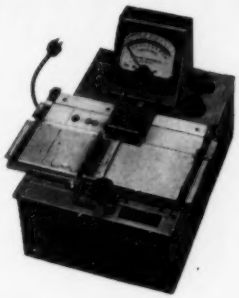

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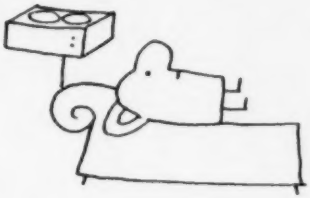

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A few minutes out of Dover, fog wrapped the flimsy Bleriot monoplane like a shroud.

The pretty young woman in the smart flying costume (she'd designed it herself—"bloomers, blouse, and hood of mauve satin") glanced at her compass. It was the first time she'd ever used one. She thought of instructor Hamel's parting words:

"Be sure to keep on course, Miss Quimby, for if you get five miles out of the way, you'll be over the North Sea, and you know what that means."

She climbed to 6,000 feet. Freezing cold and still fog.

She pointed her nose down. The comforting clatter of the Gnome engine changed to a coughing splutter. It was conking out! She leveled off, figuring how she'd ditch. To her relief, the engine suddenly took hold. Harriet re-checked her compass.

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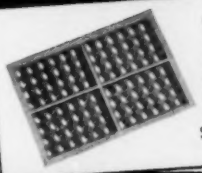

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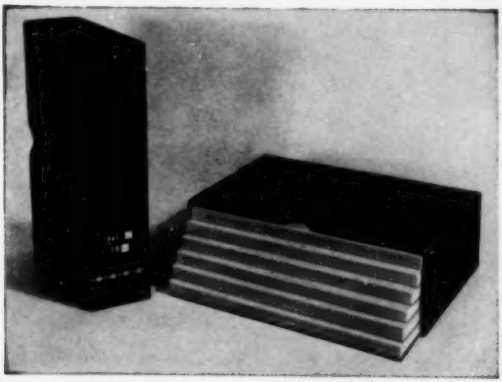

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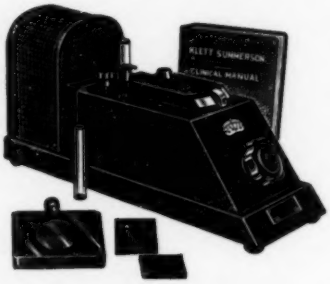

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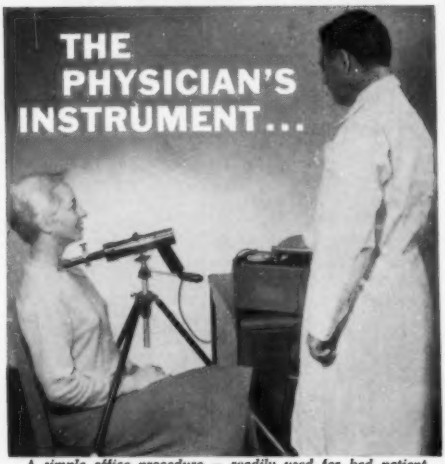

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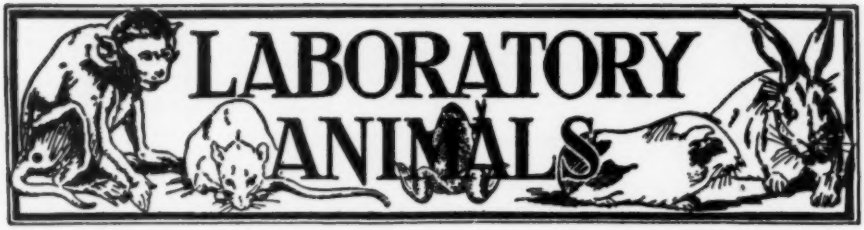

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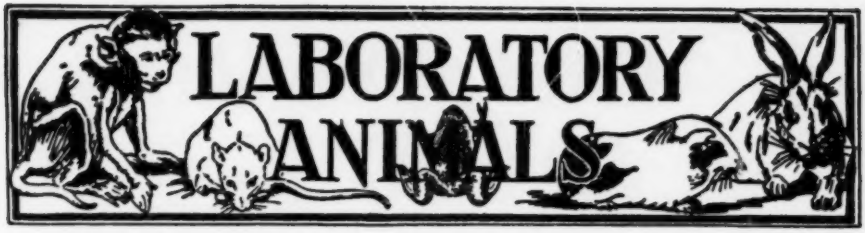

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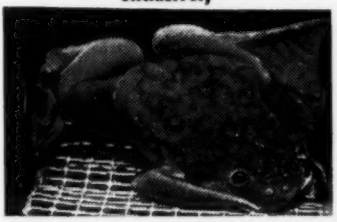

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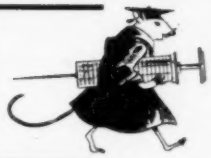

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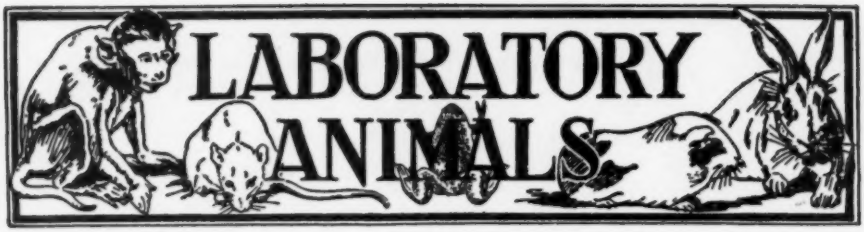

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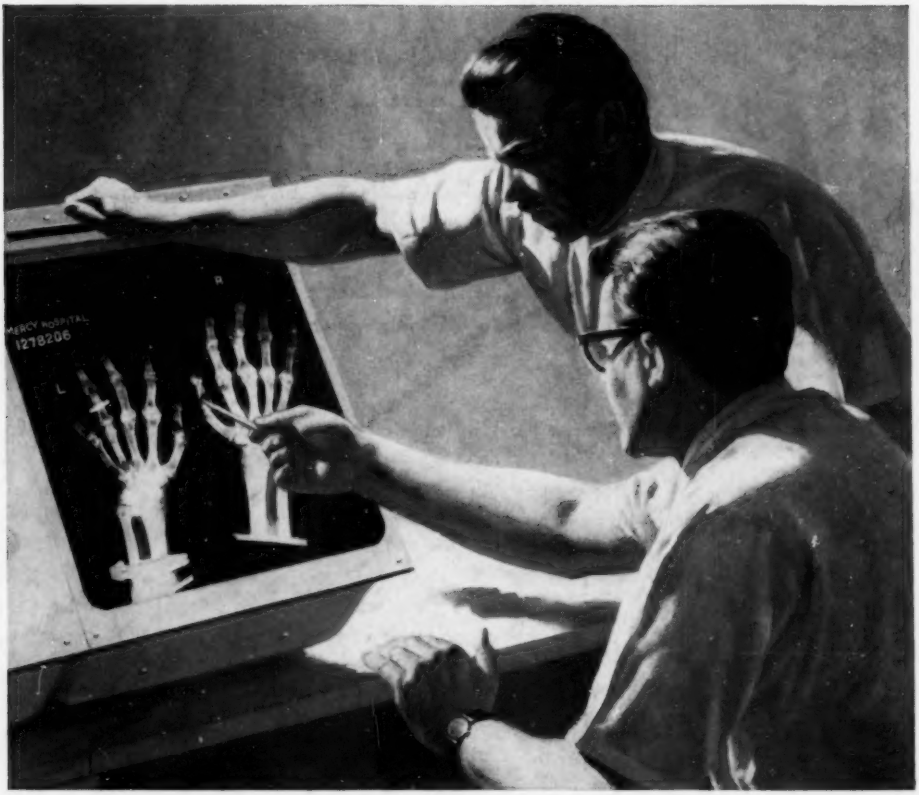

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